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<p>(21) International Application Number: PCT/GB93/01730</p> <p>(22) International Filing Date: 16 August 1993 (16.08.93)</p> <p>(30) Priority data: 9217270.9 14 August 1992 (14.08.92) GB 9304024.4 27 February 1993 (27.02.93) GB</p> <p>(71) Applicant (for all designated States except US): IMPERIAL CANCER RESEARCH TECHNOLOGY LIMITED [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB).</p>	<p>(72) Inventors; and (75) Inventors/Applicants (for US only) : VILE, Richard, Geof- frey [GB/GB]; Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 2PX (GB). HART, Ian, Roger [GB/GB]; Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX (GB).</p> <p>(74) Agent: BASSETT, Richard, S.; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).</p> <p>(81) Designated States: GB, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: TUMOUR THERAPY</p> <p>(57) Abstract</p> <p>A DNA construct comprising (i) means of expression of a coding sequence in a tumour cell and (ii) a said coding sequence encoding a cytokine. The said means for expression may provide for specific expression selectively in tumour cells, particularly melanoma cells, and pancreatic, breast, colonic and prostatic tumour cells and the cytokine is at least one of interleukin-2, interleukin-4, macrophage colony stimulating factor, interferon-γ, tumour necrosis factor and interleukin-7.</p>		

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TUMOUR THERAPY

The present invention relates to the therapy of tumours, particularly melanomas.

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Biological therapy of cancer, based upon the adoptive transfer of modified immune cells, seeks to exploit *in vivo* specificity to deliver recombinant proteins directly to the tumour mass (Parmiani *et al* (1992) *Trends Exp. Clin. Med.* 2, 412-419; Rosenberg (1992) *J. Clin. Oncol.* 10, 180-100).

10 However, this approach involves removal of cells from the patient followed by their *in vitro* manipulation and replacement *in vivo*. Proposed vaccination experiments using genetically modified tumour cells also require a similar period of passage *in vitro* during which time the neoplastic cells may significantly alter their immunological properties or
15 growth characteristics (Rosenberg (1992) *loc. cit.*; Roemer & Friedmann (1992) *Eur. J. Biochem.* 208, 211-225; Pardoll (1992) *Curr. Opin. Immunol.* 4, 619-623); Fearon *et al* (1990) *Cell* 60, 397-403.

There is experimental evidence that the expression of cytokines in tumour
20 cells (following transfection with cytokine cDNA *in vitro*) leads to rejection of otherwise tumourigenic doses of tumour cells and, in some cases, can immunise animals against established diseases when the transfected cells are injected into the animal. Cytokines shown to have this effect include interleukin-2, interleukin-4, interferon- γ , tumour
25 necrosis factor and interleukin-7. This information is summarised in Pardoll (1992) *Curr. Opinion Immunol.* 4, 619-623.

CD28-positive T cell responses, and immune responses mediated by T cells, may be regulated by the B7 antigen as described in WO 92/00092.
30 Also, tumour rejection after direct costimulation of CD8⁺ T cells by B7-

transfected melanoma cells is described in Townsend & Allison (1993) *Science* 259, 368-370.

5 Malignant melanoma represents a cancer the growth and dissemination of which may be altered significantly by immunological manipulation. Many melanomas synthesise the pigment melanin, which is otherwise produced almost exclusively by melanocytes (Hearing & Tsukamoto (1991) *FASEB J.* 5, 2902-2909) and indeed several workers have proposed utilising the melanin synthetic pathway for chemotherapeutic intervention (Riley (1991) 10 *Eur. J. Cancer* 27, 1172-1179; Link & Carpenter (1992) *Cancer Res.* 52, 4385-4390).

The tyrosinase and TRP-1 genes both encode proteins which play key roles in the synthesis of the pigment melanin, a specific product of 15 melanocytic cells. Our aim has been to utilise the 5' ends of the tyrosinase and tyrosinase-related protein (TRP-1) genes to confer tissue specificity of expression on genes cloned downstream of these promoter elements for therapeutic purposes.

20 A number of other groups already have shown that tissue specificity of expression resides within the 5' sequences of these genes (eg Bradl, M. *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88, 164-168; Jackson, I.J. *et al* (1991) *Nucleic Acids Res.* 19, 3799-3804). However we have confirmed and expanded these findings and used the promoters of these genes for 25 therapeutic purposes.

Prostate-specific antigen (PSA) is one of the major protein constituents of the human prostate secretion. It has become a useful marker for the detection and monitoring of prostate cancer. Other groups have 30 characterised the gene encoding PSA and have identified the promoter

region which directs the prostate-specific expression of PSA (Lundwall (1989) *Biochem. Biophys. Res. Comm.* 161, 1151-1159; Riegman *et al* (1989) *Biochem. Biophys. Res. Comm.* 159, 95-102; Brawer (1991) *Acta Oncol.* 30, 161-168).

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Carcinoembryonic antigen (CEA) is a widely used tumour marker, especially in the surveillance of colonic cancer patients. Although CEA is also present in some normal tissues, it is apparently expressed at higher levels in tumorous tissues than in corresponding normal tissues. The complete gene encoding CEA has been cloned and its promoter region analysed. A CEA gene promoter construct, containing approximately 400 nucleotides upstream from the translational start, showed nine times higher activity in the adenocarcinoma cell line SW303, compared with the HeLa cell line. This indicates that *cis*-acting sequences which convey cell type specific expression are contained within this region (Schrewe *et al* (1990) *Mol. Cell. Biol.* 10, 2738-2748).

The *c-erbB-2* gene and promoter have been characterised previously and the gene product has been shown to be over-expressed in tumour cell lines (Kraus *et al* (1987) *EMBO J.* 6, 605-610).

25

The mucin gene, MUC1, contains 5' flanking sequences which are able to direct expression selectively in breast and pancreatic cell lines, but not in non-epithelial cell lines as taught in WO 91/09867.

Summary of the Invention

One aspect of the invention provides a DNA construct comprising (i) means for expression of a coding sequence in a tumour cell and (ii) a said coding sequence encoding a cytokine.

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Expression of the cytokine in the tumour cells is believed to stimulate attack by T cells, especially LAK cells. Such T cells will then destroy not only the primary tumour but also any secondary (metastatic) growths.

- 5 The tumour may be a melanoma, or a tumour of the breast, colon, brain, pancreas, bladder, skin, prostate, stomach, oesophagus or liver, for example. Preferably, it is a melanoma.

- 10 Advantageously, the said means for expression provides for specific expression selectively in tumour cells. Otherwise, the T cells may attack normal cells and/or the germ line may be altered.

- By "specific expression selectively in tumour cells" we mean that the expression is usefully higher (for example 2X, 5X, 10X or at least 20X
15 higher) in tumour cells compared to the expression in non-tumour cells. It will be appreciated by those skilled in the art that tumour selective expression may be derived from tissue-specific expression where the tumour rapidly grows from a specific tissue type. Alternatively, highly specific delivery of a non-specific expression construct may be adequate.
20 Known means such as targeted liposomes (carrying anti-tumour-marker antibodies) and viruses, including retroviruses, may be employed.

- The constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses,
25 so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* 16, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers
30 are in a quiescent, non-receptive stage of cell growth. Retroviral DNA

constructs which contain a promoter segment and a cytokine coding sequence may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a *neo^R* gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μ m pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 μ g/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml. Alternatively, as described in Culver *et al* (1992) *Science* 256, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells. Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nässander *et al* (1992) *Cancer Res.* 52, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available. In relation to the present invention, antibodies directed towards tumour cell antigens such as CEA and PSA

are preferred. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

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It will be appreciated that monoclonal antibodies or other molecules that bind to tumour cell surface antigens are useful in targeting the DNA construct of the invention.

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Monoclonal antibodies which will bind to many of these antigens are already known but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to

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selected antigens may be prepared by known techniques, for example those disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982).

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Chimaeric antibodies are discussed by Neuberger *et al* (1988, 8th *International Biotechnology Symposium Part 2*, 792-799).

10 Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies. Such "humanized" antibodies, or fragments thereof, are preferred as they may give rise to a lower anti-antibody reaction than rodent antibodies.

15 The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant
20 antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving
25 the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* 240, 1041); Fv molecules (Skerra *et al* (1988) *Science* 240, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988)
30 *Science* 242, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85,

5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* 341, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* 349, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

10 The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be
15 expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen
20 combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

Other molecules immunologically reactive with the target cell surface molecule are also useful in this aspect of the invention and include, for
25 example minimal recognition units (MRU) and complementarity determining regions.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* 40, 1-18) and
30 transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc.*

Natl. Acad. Sci. USA 87, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA via electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalized into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

10

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

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High-efficiency receptor-mediated delivery of the DNA constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* 89, 6094-6098 may also be used. This

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approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct of the invention, the DNA construct is taken up by the cell
5 by the same route as the adenovirus particle.

It may be desirable to locally perfuse a tumour with the delivery vehicle (for example the retrovirus) for a period of time.

- 10 In one embodiment of the invention the said means for expression provides for specific expression selectively in melanoma cells or in melanoma cells and melanocytes. In this embodiment the said means for expression is a promoter or an analogue or part thereof forming part of a gene expressed substantially exclusively in the melanin synthesis pathway.
15 Examples of such promoters include the tyrosinase gene promoter and the tyrosinase-related protein (TRP-1) gene promoter.

By "promoter" we mean that region of DNA which controls, at least to a substantial extent, the transcription of the coding region associated with
20 that region of DNA.

In a further embodiment of the invention the said means for expression provides for specific expression selectively in prostate cancer cells or prostate cancer cells and prostate cells. In this embodiment the said
25 means for expression is a promoter or an analogue or part thereof forming part of a gene expressed substantially exclusively in prostate cancer or prostate cells. An example of such a promoter is the prostate-specific antigen (PSA) gene promoter.

30 In a still further embodiment of the invention the said means for

expression provides for specific expression selectively in colonic cancer cells, or colonic cancer cells and colon cells. In this embodiment the said means for expression is a promoter or an analogue or part thereof forming part of a gene expressed substantially exclusively in colon cancer or colon cells. An example of such a promoter is the carcinoembryonic antigen (CEA) gene promoter.

In another embodiment of the invention the said means for expression is provided by the promoter region of the *c-erbB2*-gene.

10

In this embodiment the constructs comprising the *c-erbB2* gene promoter fused to the cytokine coding sequence may be usefully delivered to breast tumours. The *c-erbB3* gene promoter may also be used.

15 In yet another embodiment the said means for expression is provided by the promoter region of the *MUC1* gene.

In this embodiment pancreatic or breast tumours may usefully receive the constructs comprising *MUC1* gene promoter fused to the cytokine coding sequence.

20

DNA sequences encompassing the promoter sequences useful in the invention are given in the sequence listing.

25 The cytokine is preferably interleukin-2 or interleukin-4 or macrophage colony stimulating factor. Other cytokines may, however, be used, for example interferon- γ , tumour necrosis factor, and interleukin-7. Nucleotide coding sequences for these are known and are given in the sequence listing.

30

The promoter is joined to the cytokine coding region and placed in a suitable vector system for propagation. The skilled person can use the information given below containing the promoter DNA sequences and coding sequences of some of the cytokines useful in the invention to make
5 suitable constructs. For example, a knowledge of the DNA sequences provides information on where restriction enzyme will cleave the said DNA molecules and allows oligonucleotide primers to be designed for PCR amplification and site-directed mutagenesis.

10 The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the vector a DNA sequence, with any necessary control elements, that codes for a selectable
15 trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA construct
20 of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the propagation of the DNA construct, which can then be recovered.

25 The vectors usually include a procaryotic replicon, such as the ColE1 *ori*, for propagation in a procaryote, even if the vector is to be used for expression in other, non-procaryotic, cell types.

It is preferred if the host cell is *E. coli*.

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then
5 joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA
10 segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

15 The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as
20 bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

25 Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

30 DNA fragments with complementary cohesive termini are readily joined

together by ligation using methods known in the art and described in Sambrook *et al* (1989) *Molecular Cloning, A laboratory manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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A desirable way to modify the promoter fragment, vector or coding region to be fused in the DNA construct is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491.

- 10 In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

15

- The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell for propagating the DNA construct can be either procaryotic or eucaryotic. Bacterial cells are preferred host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343).

- 25 Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of bacterial, especially *E. coli* host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor
- 30

Laboratory, Cold Spring Harbor, NY.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208 or by isolating the plasmid vector DNA and then digesting the said plasmid appropriate restriction enzymes that give diagnostic DNA fragments that can be separated and sized by gel electrophoresis.

The DNA construct of the invention is purified from the host cell using well known methods.

For example, plasmid vector DNA can be prepared on a large scale from cleaved lysates by banding in a CsCl gradient according to the methods of Clewell & Helinski (1970) *Biochemistry* 9, 4428-4440 and Clewell (1972) *J. Bacteriol.* 110, 667-676. Plasmid DNA extracted in this way can be freed from CsCl by dialysis against sterile, pyrogen-free buffer through Visking tubing or by size-exclusion chromatography.

Alternatively, plasmid DNA may be purified from cleared lysates using ion-exchange chromatography, for example those supplied by Qiagen. Hydroxyapatite column chromatography may also be used.

25

Preferably, naked DNA is injected in the tumour, for example at a dose of 0.1 ng to 1.0 mg vector DNA cm⁻³ of tumour, preferably about 0.1-10 µg cm⁻³ vector DNA. The DNA may be circular or linear. Linear DNA may be obtained from circular DNA by cleavage with an appropriate restriction enzyme.

30

By "appropriate restriction enzyme" we mean one that does not cleave the DNA within the promoter region or cytokine coding region.

At present, it is most preferable to use 1.0 μg of DNA per cm^3 of tumour
5 in a volume of 100 μl . The DNA may be dissolved in phosphate-buffered
saline (PBS), or it may be used as a precipitate with calcium phosphate.
Of course, other suitable buffers or carriers may usefully be employed.
The expression of the said DNA in the tumour may be analysed by reverse
transcriptase-PCR (that is, the messenger RNA expressed from the DNA
10 in the tumour is isolated, converted into complementary DNA (cDNA)
using the enzyme reverse transcriptase, and the resultant cDNA is
amplified using the polymerase chain reaction and may be detected
radiolabelling or staining), or by northern blot analysis or by RNase
protection assays.

15

Such injection may be repeated at hourly, daily or weekly intervals.

Uptake of naked DNA may depend on the three-dimensional growing mass
of tumour so, although it is preferred that the tumour to be treated is
20 melanoma, a prostate tumour, or a colon tumour or a pancreatic tumour,
or a breast tumour, it may be any solid tumour.

It is most preferred if substantially all cells in the tumour take up DNA
and express the cytokine, but it is not essential for a useful clinical effect,
25 as the antitumour effect of the cytokine is not limited to the tumour cell
expressing the cytokine but will occur in non-transfected cells within the
tumour and at secondary (metastatic) sites. Thus, if 5%, preferably 25%,
more preferably 50% and most preferably substantially 100% of the
tumour cells express the cytokine a clinically useful effect may be seen.

30

It is desirable to express a plurality of cytokine coding sequences in a tumour cell, or to express a plurality of cytokine coding sequences in a tumour wherein each cytokine coding sequence is present in a separate DNA construct. It is preferable if the different cytokines, expressed by the plurality of coding sequences, stimulate different effector cells of the immune system.

In one embodiment, each of the coding sequences of the plurality are directly joined to a means for expression in a tumour cell but are contained within the same DNA construct. Thus, once the DNA is introduced into the tumour, every cell that takes up the DNA may express all of the cytokine coding sequences in the plurality.

In a further embodiment, a plurality of DNA constructs is introduced into the tumour, each construct of the plurality comprises a means for expression of a coding sequence in a tumour cell and a coding sequence encoding a different cytokine. In this embodiment it is possible to vary the proportion of cytokine coding sequences in the plurality.

The components of the plurality comprise two or more of coding sequences encoding interleukin-2, interleukin-4, macrophage colony stimulating factor, interferon- γ , tumour necrosis factor and interleukin-7. The ratio of any two of the said coding sequences in the plurality may be, one to another, 100:1, 10:1 or 1:1.

25

Thus, a particular plurality of coding sequences useful in the invention is interleukin-2:interleukin-4:macrophage colony stimulating factor in a molar ratio of 1:1:1. This particular combination of coding sequences will express a plurality of cytokines useful in attracting cytotoxic T cells, eosinophils and macrophages to the tumour, and to secondary (metastatic)

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sites. All of these cell types have been shown to have anti-tumour activity.

5 It is preferred that the means of expressing each coding sequence in the plurality is a tumour specific promoter.

It is preferred that the plurality of DNA constructs is injected directly into the tumour.

10 It is further preferred that the tumour into which the DNA construct is injected directly is a melanoma, breast cancer, prostate cancer or colon cancer.

15 It is desirable to treat melanoma with a DNA construct wherein the means of expressing is the tyrosinase promoter.

In a further embodiment it is preferred if the B-cell accessory molecule B7 antigen is co-expressed with the cytokine in the tumour or tumour cell. B7 binds CD28 on T-cells and stimulates the activity of T-cells against
20 tumours as is described in WO 92/00092.

The cDNA encoding the B7 antigen molecule can be obtained using the method described by Freeman *et al* (1989) *J. Immunol.* 143, 2714-2722 incorporated herein by reference and the nucleotide and predicted amino
25 acid sequence can be obtained therefrom. The nucleotide sequence of B7 cDNA is given as SEQ ID No 23.

The term "fragment" as used herein means a portion of the amino acid sequence corresponding to the B7 antigen. For example, a fragment of
30 the B7 antigen useful in the method of the present invention is a

polypeptide containing a portion of the amino acid sequence corresponding to the extracellular portion of the B7 antigen, ie the DNA encoding amino acid residues from position 1 to 215 of the sequence corresponding to the B7 antigen described by Freeman *et al, supra*.

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Complementary cDNA sequences encoding the amino acid sequence corresponding to the B7 antigen or fragments or derivatives thereof can be synthesised by the polymerase chain reaction (see US Patent No 4,683,202) using primers derived from the published sequence of the antigen (Freeman *et al, supra*). These cDNA sequences can then be assembled into a vector so that the expression of the B7 antigen is driven by a means for expression in the tumour cell.

15

It is preferred if the means for expression is a tumour-specific promoter.

It is further preferred if the promoter is the tyrosinase or TRP-1 promoter.

It is preferred if the tumour is melanoma.

20 The techniques for assembling and expressing DNA encoding the amino acid sequences corresponding to B7 antigen and the cytokines useful in the invention, eg synthesis of oligonucleotides, PCR, transforming cells, constructing vectors and the like are well-established in the art, and most practitioners are familiar with the standard resource materials for specific conditions and procedures. However, the following paragraphs are provided for convenience and notation of modifications where necessary, and may serve as a guideline.

30

Complementary cDNA clones containing DNA encoding B7 proteins are obtained to provide DNA for assembling into the DNA constructs for use

in the methods of the invention. Alternatively, cDNA clones may be prepared from RNA obtained from cells expressing B7 antigen or the cytokines based on knowledge of the published sequences for these proteins using standard procedures. Published sequences for the cDNAs
5 are given as SEQ ID Nos.

The cDNA is amplified using the polymerase chain reaction ("PCR") technique (see US Patent Nos. 4,683,195 and 4,683,202 to Mullis *et al* and Mullis & Faloona (1987) *Methods Enzymol.* 154, 335-350) using
10 synthetic oligonucleotides encoding the sequences of the proteins as primers. PCR is then used to adapt the fragments for ligation to the DNA encoding the promoter fragments and to expression plasmid DNA to form cloning and expression plasmids.

15 It is desirable to express a single cytokine coding sequence or a plurality of cytokine coding sequences in a tumour cell, in combination with the B7 coding sequence, or to express a cytokine coding sequence in a tumour in combination with a B7 coding sequence wherein the cytokine coding sequence and the B7 coding sequence are present in a separate DNA
20 construct. It is preferable if the different cytokines, expressed by the plurality of coding sequences, stimulate different effector cells of the immune system.

In one embodiment, each of the coding sequences of the plurality of
25 cytokines or B7 coding sequence are directly joined to a means for expression in a tumour cell but are contained within the same DNA construct. Thus, once the DNA is introduced into the tumour, every cell that takes up the DNA may express all of the cytokine coding sequences in the plurality and the B7 coding sequence.

In a further embodiment, a plurality of DNA constructs is introduced into the tumour, each construct of the plurality comprises a means for expression of a coding sequence in a tumour cell or a coding sequence encoding a different cytokine or B7 molecule. In this embodiment it is possible to vary the proportion of cytokine coding sequences and B7 molecules introduced into the tumour.

It will be appreciated by one skilled in the art that the same or different cytokine or B7 coding sequence may be expressed in the tumour cell from separate DNA constructs or that the said coding sequences may be expressed in the tumour cell from the same DNA construct wherein each coding sequence has an independent means for expression or that the said coding sequences may be expressed in the tumour cell from the same DNA construct wherein each coding sequence has the same means for expression. In the latter case the coding sequences for a cytokine or a B7 may be fused such that a fusion polypeptide is made; it is preferred if a linker joins the polypeptides in the fusion that is cleaved in the environment of the tumour cell to release the active cytokine or B7.

When melanoma is to be treated by the DNA constructs comprising a gene promoter from a melanin synthesis pathway gene such as tyrosinase, it is desirable if the patient to be treated is not black.

It is further preferred if the patient to be so treated is fair-skinned.

In a further aspect of the invention the DNA constructs are used in conjunction with chemotherapy. Thus, the DNA construct, or a plurality of such constructs, may be administered at the same time as, preceding or after treatment with chemotherapeutic agents.

Chemotherapeutic agents useful in this aspect of the invention include cisplatin, dacarbazine, tamoxifen, nitrosoureas including carmustine (BCNU), vinca alkaloids, melphalan, doxorubicin, adriamycin, etoposide, 5-fluorouracil and other generally used agents.

5

These are listed in the table:

TABLE: CHEMOTHERAPEUTIC AGENTS

Class	Type of Agent	Nonproprietary Names (Other Names)
Alkylating Agents	Nitrogen Mustards	Mechlorethamine (HN ₂)
		Cyclophosphamide Ifosfamide
		Melphalan (L-sarcolysin)
		Chlorambucil
	Ethylenimines and Methylmelamines	Hexamethylmelamine
		Thiotepa
	Alkyl Sulfonates	Busulfan
	Nitrosoureas	Carmustine (BCNU)
		Lomustine (CCNU)
		Semustine (methyl-CCNU)
		Streptozocin (streptozotocin)
	Triazenes	Decarbazine (DTIC; dimethyltriazenoimidazolecarboxamide)

5

10

Class	Type of Agent	Nonproprietary Names (Other Names)
Antimetabolites	Folic Acid Analogs	Methotrexate (amethopterin)
	Pyrimidine Analogs	Fluorouracil (5-fluorouracil; 5-FU) Flouxuridine (fluorodeoxyuridine; FUdR)
		Cytarabine (cytosine arabinoside)
Antimetabolites continued	Purine Analogs and Related Inhibitors	Mercaptopurine (6-mercaptopurine; 6-MP)
		Thioguanine (6-thioguanine; TG)
		Pentostatin (2'-deoxycoformycin)

5

10

15

20

Class	Type of Agent	Nonproprietary Names (Other Names)
Natural Products	Vinca Alkaloids	Vinblastine (VLB)
		Vincristine
	Epipodophyl- lotoxins	Etoposide
		Teniposide
	Antibiotics	Dactinomycin (actinomycin D)
		Daunorubicin (daunomycin; rubidomycin)
		Doxorubicin
		Bleomycin
		Plicamycin (mithramycin)
		Mitomycin (mitomycin C)
	Enzymes	L-Asparaginase
	Biological Response Modifiers	Interferon alfa
Miscellaneous Agents	Platinum Coordination Complexes	Cisplatin (<i>cis</i> -DDP) Carboplatin
	Anthracenedione	Mitoxantrone
	Substituted Urea	Hydroxyurea
	Methyl Hydrazine Derivative	Procarbazine (N-methylhydrazine, MIH)
	Adrenocortical Suppressant	Mitotane (<i>o,p'</i> -DDD)
		Aminoglutethimide

It is preferred if the DNA construct or the plurality of constructs expresses interleukin-2 which will facilitate the substantial destruction of the vasculature and promote the action of the chemotherapeutic agent.

- 5 Further aspects of the invention provide a composition comprising a construct of the invention and means for selectively delivering it to a tumour and a method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a construct of the invention.

10

The invention will now be described with reference to the following Examples and Figures wherein:

- Figure 1 shows the tissue specific expression cassettes using the tyrosinase and the TRP-1 gene promoters;

15

Figure 2 shows the relative activity of tyrosinase and TRP-1 promoters in murine B16.F1 melanoma and NIH 3T3 cells;

- 20 Figure 3 shows the retroviral vector pBabe Puro (Tyr- β -Gal).

Figure 4 shows the *c-erbB-2*/CAT construct of Example 5.

- Figure 5 shows the result of a comparison of activity of the construct of Example 5 in two cell lines: T47D, which is a breast carcinoma cell line with base line *c-erbB-2* expression, and ZR75-1, which is a breast carcinoma cell line with elevated *c-erbB-2* expression.

25

- SEQ ID No 1 shows the nucleotide sequence of the CEA gene including the promoter region.

30

SEQ ID No 2 shows the sequence of the PSA gene including the promoter region.

5 Figure 6 shows the 5' flanking sequence with 71 bp of transcribed sequence of the human MUC1 gene (SEQ ID No 3). The TATA box (boxed) and transcriptional start site (+1) are indicated. The sequence (-787 to +71) covers the region required for maximum transcription of the reporter gene (-743 to +33).

10 Figure 7 shows the DNA sequence of the human *c-erbB-2* 5' region as determined by Hudson *et al* (1990) *J. Biol. Chem.* 265, 4389-4393 (SEQ ID No 4).

15 Figure 8 shows the DNA sequence of the human *c-erbB-3* 5' region (SEQ ID No 5) and the predicted amino acid sequence of the first exon (SEQ ID No 6).

SEQ ID No 7 shows the DNA sequence of the tyrosinase promoter.

20 SEQ ID No 8 shows the DNA sequence of the TRP-1 promoter.

SEQ ID No 9 shows the DNA gene sequence encoding interleukin-2 (IL-2); the cDNA sequence is readily derived from the positions of the exons.

25 SEQ ID No 10 shows the cDNA sequence encoding interleukin-4 (IL-4).

SEQ ID No 11 shows the cDNA sequence encoding interleukin-7 (IL-7).

30 SEQ ID No 12 shows the cDNA sequence encoding tumour necrosis factor (TNF).

SEQ ID No 21 shows the cDNA sequence encoding interferon-gamma (IFN- γ).

SEQ ID No 22 shows the cDNA sequence encoding human granulocyte
5 macrophage colony stimulating factor GM-CSF.

SEQ ID No 23 shows the B7 cDNA sequence.

The following information is useful to the person skilled in the art to
10 identify coding regions and promoter sequences for use in the invention.
Journal references and EMBL database accession numbers are given.

SEQ ID No 1

15 ID HSCEA01 standard; DNA; PRI; 3281 BP; AC M59255; M31966; DE
Human carcinoembryonic antigen (CEA) gene, complete cds; KW
carcinoembryonic antigen; OS Homo sapiens (human); OC Eukaryota;
Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria;
Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP
20 1-3281; RA. Schrewe H., Thompson J., Bona M., Hefta L.J., Maruya
A., RA Hassauer M., Shively J.E., von Kleist S., Zimmermann W; RT
"Cloning of the complete gene for carcinoembryonic antigen;; RT
Analysis of its promoter indicates a region conveying cell; RT
type-specific expression"; RL Mol. Cell. Biol. 10:2738-2748(1990); FH
25 Key Location/Qualifiers; FH; FT sig_peptide
join(1769..1832,2725..2762); FT /gene="CEA"; FT exon 1659..1832;
FT /number=1 /gene="CEA" /codon_start=1659; FT exon 2725..3084;
FT /number=2 /gene="CEA" /codon_start=2725; SQ Sequence 3281
BP; 847 A; 953 C; 871 G; 610 T; 0 other; CC

SEQ ID No 21

ID HSIFNGAMM standard; RNA; PRI; 1011 BP; AC M26683; DT
23-NOV-1989 (Rel. 21, Created); DT 26-MAY-1992 (Rel. 32, Last
5 updated, Version 5); DE Human interferon gamma (IFN-gamma) mRNA,
complete cds; KW interferon gamma; type II; OS Homo sapiens (human);
OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC
Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN. [1];
RP 1-1011; RA Fan X., Stark G.R., Bloom B.R; RT "molecular cloning
10 of a gene selectively induced by gamma; RT interferon from human
macrophage cell line u937"; RL Mol. Cell. Biol. 9:1922-1928(1989); FH
Key Location/Qualifiers; FH; FT CDS 15..131; FT /product="interferon
gamma" /gene="IFN-gamma"; FT /codon_start=1; FT polyA_signal
971..976; FT /gene="IFN-gamma"; SQ Sequence 1011 BP; 301 A; 236
15 C; 184 G; 290 T; 0 other;

SEQ ID No 2

ID HSPSAA standard; DNA; PRI; 7130 BP; AC M27274; DT
20 23-APR-1990 (Rel. 23, Last updated, Version 1); DT 02-FEB-1990 (Rel.
22, Created); DE Human prostate-specific antigen gene, complete cds;
KW Alu repetitive element; kallikrein; prostate specific antigen; OS Homo
sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata;
Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini;
25 Catarrhini; Hominidae; RN [1]; RP 1-7130; RA Lundwall A; RT.
"Characterization of the gene for prostate-specific antigen, a; RT human
glandular kallikrein"; RL Biochem. Biophys. Res. Commun.
161:1151-1159(1989); DR SWISS-PROT; P07288; PROS\$HUMAN; FH
Key Location/Qualifiers; FH; FT CDS 675..720; FT
30 /note="prostate-specific antigen, exon 1; FT /nomgen="APS"

/map="19q13.3-qter"; FT /hgml_locus_uid="LN0098S"; FT intron
721..1958; FT /note="PSA intron A"; FT CDS 1959..2118; FT
/note="prostate-specific antigen, exon 2"; FT intron 2119..3755; FT
/note="PSA intron B"; FT repeat_region 2583..2935; FT /note="Alu
5 repeat"; FT CDS 3756..4042; FT /note="prostate-specific antigen, exon
3"; FT intron 4043..4185; FT /note="PSA intron C"; FT CDS
4186..4322; FT /note="prostate-specific antigen, exon 4"; FT intron
4323..5698; FT /note="PSA intron D"; FT CDS 5699..5854; FT
/note="prostate-specific antigen, exon 5"; SQ Sequence 7130 BP; 1530
10 A; 2024 C; 1867 G; 1709 T; 0 other;

SEQ ID No 8

ID MMTRP15 standard; DNA; ROD; 1236 BP; AC X59513; DT
15 26-JUL-1991 (Rel. 28, Created); DT 26-JUL-1991 (Rel. 28, Last updated,
Version 2); DE Mouse 5' end of TRP1 gene for tyrosinase-related
protein-1; KW TRP1 gene; tyrosinase; tyrosinase-related protein-1; OS
Mus musculus (mouse); OC Eukaryota; Animalia; Metazoa; Chordata;
Vertebrata; Mammalia; OC Theria; Eutheria; Rodentia; Myomorpha;
20 Muridae; Murinae; RN. [1]; RA Jackson I.J., Chambers D.M., Budd
P.S., Johnson R; "The tyrosinase-related protein-1 gene has a structure
and promoter sequence very different from tyrosinase."; Nucleic Acids
Res. 19:3799-3804(1991) SQ Sequence 1236 BP; 357 A; 234 C; 282 G;
363 T; 0 other;

25

SEQ ID No 22

ID HSCSFGMA standard; DNA; PRI; 3194 BP; AC M13207; DT
07-JUN-1987 (Rel. 12, Created); DT 24-DEC-1990 (Rel. 26, Last
30 updated, Version 2); DE Human granulocyte-macrophage

colony-stimulating factor (hGM-CSF); DE gene, complete cds; KW granulocyte-macrophage colony stimulating factor; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; 5 Hominidae; RN [1]; RP 1-3194; RA Kaushansky K., O'Hara P.J., Berkner K., Segal G.M., Hagen F.S.,; RA Adamson J.W; RT "Genomic cloning, characterization, and multilineage; RT growth-promoting activity of human granulocyte-macrophage; RT colony-stimulating factor"; RL Proc. Natl. Acad. Sci. U.S.A. 83:3101-3105(1986); RN [2]; RP 1-3194; 10 RA Kaushansky K; RT; RL Unpublished; DR CPGISLE; HSCSFGMA; Release pre-1.0; DR SWISS-PROT; P04141; CSF2_HUMAN; SQ Sequence 3194 BP; 700 A; 859 C; 945 G; 690 T; 0 other; CC

SEQ ID No 9

15

ID HSIL21 standard; DNA; PRI; 5737 BP; AC J00264; DT 29-JUL-1991 (Rel. 28, Created); DT 29-JUL-1991 (Rel. 28, Last updated, Version 1); DE Human interleukin 2 (IL-2) gene, complete coding sequence; KW immune response gene; interleukin; interleukin 2; lymphokine; KW T-cell; 20 T-cell growth factor; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 431-624, 715-774, 3068-3211, 5057-5443; RA Maeda S., Nishino N., Obaru K., Mita S., Nomiyama H., Shimada K.,; RA Fujimoto K., 25 Teranishi T., Hirano T., Onoue K; RT "Cloning of interleukin 2 mRNAs from human tonsils"; RL Biochem. Biophys. Res. Commun. 115:1040-1047(1983); RN CC Key Location/Qualifiers; FH; FT CDS join (478..624,715..774,3068..3211,5057..5167); SQ Sequence 5737 BP; 1995 A; 932 C; 922 G; 1888 T; 0 other; CC; ID HSIL4 standard; RNA; PRI; 30 614 BP; AC M13982; DT 07-JUN-1987 (Rel. 12, Created); DT

03-SEP-1992 (Rel. 33, Last updated, Version 2);

SEQ ID No 10

5 KW interleukin; OS Homo sapiens (human); OC Eukaryota; Animalia;
Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria;
Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-614; RA
Yokota T., Otsuka T., Mosmann T., Banchereau J., DeFrance T., RA
Blanchard D., De Vries J.E., Lee F., Arai K.i."Isolation and
10 characterization of a human interleukin cDNA clone homologous to mouse
B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating
activities"Proc. Natl. Acad. Sci. U.S.A. 83:5894-5898(1986). ; DR
SWISS-PROT; P05112; IL4_HUMAN; FH Key Location/Qualifiers; FH;
FT mRNA <1..614; FT /note="IL-4 mRNA"; FT CDS 64..524; FT
15 /note="interleukin 4" /gene="IL4" /partial; FT sig_peptide 64..135; FT
/note="interleukin 4 signal peptide"; FT mat_peptide 136..522; FT
/note="interleukin 4 mature peptide"; SQ Sequence 614 BP; 174.A; 150
C; 129 G; 161 T; 0 other;

20 SEQ ID No 11

ID HSIL7A standard; RNA; PRI; 1589 BP; AC J04156; DT
22-APR-1989 (Rel. 19, Created); DT 06-JUL-1989 (Rel. 20, Last
updated, Version 1); DE Human interleukin 7 (IL-7) mRNA, complete
25 cds; KW interleukin; interleukin 7; OS Homo sapiens (human); OC
Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC
Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1];
RP 1-1589; RA Goodwin R.G., Lupton S., Schmieder A., Hjerrild K.J.,
Jerzy R., RA Clevenger W., Gillis S., Cosman D., Namen A.E; RT
30 "Human interleukin 7: Molecular cloning and growth factor activity; RT

on human and murine B-lineage cells"; RL Proc. Natl. Acad. Sci. U.S.A. 86:302-306(1989); DR SWISS-PROT; P13232; IL7_HUMAN; CC Draft entry and computer-readable sequence [1] kindly submitted by; CC R.Goodwin, 05-JAN-1989; FH Key Location/Qualifiers; FH; FT mRNA <1..1589; FT /note="interleukin 7 mRNA"; FT CDS 385..918; FT /note="interleukin 7 precursor"; FT CDS 385..459; FT /note="interleukin 7 signal peptide"; FT CDS 460..915; FT /note="interleukin 7"; SQ Sequence 1589 BP; 532 A; 284 C; 339 G; 434 T; 0 other;

10

SEQ ID No 12

Human tumour necrosis factor mRNA; ID HSTNFAA standard; RNA; PRI; 1585 BP; AC M10988; DT 16-JUL-1988 (Rel. 16, Created); DT 15 02-SEP-1992 (Rel. 33, Last updated, Version 2); DE Human tumor necrosis factor (TNF) mRNA; KW ; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-1585; RA Wang A.M., Creasey A.A., Ladner M.B., Lin L.S., 20 Strickler J.,; RA Van Arsdel J.N., Yamamoto R., Mark D.F; RT "Molecular cloning of the complementary DNA for human tumor; RT necrosis factor"; RL Science 228:149-154(1985); DR SWISS-PROT; P01375; TNFA_HUMAN; FH Key Location/Qualifiers; FH; FT CDS 86..787; FT /note="tumor necrosis factor" /gene="TNFA"; FT 25 /codon_start=1; SQ Sequence 1585 BP; 352 A; 473 C; 389 G; 371 T; 0 other; CC

SEQ ID No 7

30 ID MMTYR1 standard; DNA; ROD; 4758 BP; AC D00439; DT

14-FEB-1991 (Rel. 27, Created); DT 14-FEB-1991 (Rel. 27, Last updated, Version 1); DE Mouse tyrosinase gene, 5'flank and exon 1; KW melanin; melanocyte; monooxygenase; tyrosinase; OS Mus musculus (mouse); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Rodentia; Myomorpha; Muridae; Murinae; RN [1]; RP 2481-3363; RA ; RN [2]; RP 1-4758; RA Yamamoto H., Takeuchi S., Kudo T., Sato C., Takeuchi T; RT "Melanin production in cultured albino melanocytes transfected; RT with mouse tyrosinase cDNA"; RL Jpn. J. Genet. 64:121-135(1989); FH Key Location/Qualifiers; FH; FT misc_signal 2004..2008; FT /note="putative CAT box"; FT misc_signal 2128..2133; FT /note="putative CAT box"; FT misc_signal 2140..2146; FT /note="putative TATA box"; FT misc_signal 2264..2268; FT /note="putative CAT box"; FT misc_signal 2272..2279; FT /note="putative TATA box"; FT misc_signal 2286..2289; FT /note="putative CAT box"; FT misc_signal 2434..2440; FT /note="putative TATA box"; FT misc_feature 2465..2466; FT /note="CAP sites"; FT CDS 2545..>3363; FT /note="tyrosinase gene, exon 1" /partial; SQ Sequence 4758 BP; 1550 A; 859 C; 878 G; 1465 T; 6 other; CC

20

SEQ ID No 23

; ID HSIGB7 standard; RNA; PRI; 1491 BP. ; AC M27533; ; DT 23-APR-1990 (Rel. 23, Created) ; DT 23-APR-1990 (Rel. 23, Last updated, Version 1) ; DE Human Ig rearranged B7 protein mRNA VC1-region, complete cds. ; KW constant region; immunoglobulin; variable region. ; OS Homo sapiens (human) ; OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; ; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae. ; RN [1] ; RP 1-1491 ; RA Freeman G.J., Freedman A.S., Segil J.M., Lee G., Whitman J.F., ; RA

30

Nadler L.M.; ; RT "B7, a new member of the Ig superfamily with unique expression on ; RT activated and neoplastic B cells"; ; RL J. Immunol. 143:2714-2722(1989). ; CC Draft entry and computer readable copy of sequence [1] kindly ; CC provided by G.J. Freeman, 08-SEP-1989. ; FH

5 Key Location/Qualifiers ; FH ; FT CDS 318..1184 ; FT /note="transmembrane protein B1 precursor" ; FT CDS 318..395 ; FT /note="transmembrane protein B1 signal ; FT peptide" ; FT CDS 396..1181 ; FT /note="transmembrane protein B1" ; SQ Sequence 1491 BP; 419 A; 343 C; 311 G; 418 T; 0 other; ; CC

10

Example 1: Demonstration of tissue specificity of 5' sequences of murine tyrosinase and TRP-1 genes.

A 2.5kb fragment from the 5' end of the tyrosinase gene was generated

15 by PCR from genomic DNA of the B16 melanoma line. The oligonucleotides used (Pair 1: 5'-CGGAATTTCATGCCCCAGTTGAC-AACATAG-3', SEQ ID No 13; 5'-CACTCGAGAACTTTTCTCCTTTAGATCATACAA-3', SEQ ID No 14) were derived from the murine sequence published by Yamamoto *et al* (1989) *Jpn. J. Genet.* 64, 121-135.

20 Shorter 5' sequences were generated also using oligonucleotides matched from the Yamamoto paper (Pair 2: 5'-CGGGAATTTCATGCCCCAGTTGACAACATAG-3', SEQ ID No 15; 5'-GAGCTCGAGTGTACAGACTTCTTTTCCA-3, SEQ ID No 16; Pair 3: 5'-AAACGAATTCCATCCAGTAAGTCCATTACT-3', SEQ ID No

25 17; 5'-GAGCTCGAGTGTACAGACTTCTTTC-3', SEQ ID No 18). The 769bp fragment of the tyrosinase gene extends from position -815 to position -46 in the promoter. A 4.0kb fragment of 5' sequence of the TRP-1 gene was provided by Dr I.J. Jackson, MRC Genetics Unit, Edinburgh and from this a 1.4kb fragment was derived by *Xba*I-*Sal*I

30 digestion. The promoter sequence at the 5' of TRP-1 gene may be

obtained following the methods described in Jackson *et al* (1991) *Nucl. Acids Res.* 19, 3799-3804.

5 These 5' sequences, and the SV40 promoter as a control, were inserted upstream of the β -galactosidase gene in the vector pNASS (obtained from Clontech Ltd) as indicated in Figure 1.

Figure 1 shows (A) pNASS β , a promoterless mammalian expression vector described by MacGregor & Caskey (1989) *Nucl. Acids Res.* 17, 10 2365. Three unique restriction sites allow cloning of promoter sequences upstream of an expression cassette containing the SV40 splice donor/acceptor sequence (sd/sa), the β -galactosidase gene and the SV40 polyadenylation sequence. SV40 β -Gal contains the SV40 early viral promoter (from the pBabe Puro vector, as described by Morgenstern & 15 Land (1990) *Nucl. Acids Res.* 18, 3596, cloned into pNASS β . (B) 2496 bp (Tyr- β -Gal 1) or 769 bp (Tyr- β -Gal 2) fragments of the mouse tyrosinase promoter (Yamamoto *et al* (1989) *Jap. J. Genet.* 64, 121-135) were generated by PCR from genomic DNA of the B16.F1 melanoma cell line and cloned into the *EcoRI* and *XhoI* restriction sites of pNASS β . (C) 20 The plasmids TRP-1- β -Gal 1 and 2 were a gift from I. Jackson and contain 4 kbp and 1.4 kbp of the TRP-1 promoter (Jackson *et al* (1991) *Nucl. Acids Res.* 19, 3798-3804) upstream of the β -galactosidase gene and the SV40 polyadenylation sequence. The different constructs were transfected into a variety of murine and human cells of melanocytic and 25 non-melanocytic origin, including B16 melanoma cells or NIH 3T3 fibroblasts and subsequent β -galactosidase activity was measured 72-96 hours after transfection both by fluorometric assay, using 4-methylumbelliferyl- β -D-galactoside (MUG) as substrate, and by histochemical analysis using X-gal as substrate. By both assays the 30 various tyrosinase and TRP-1 promoter containing 5' sequences were

shown to drive β -galactosidase activity in a murine melanocyte (Mel-ab) line and the B16 melanoma and the human melanoma lines SK23, HMB-2, Mel 8, TXM13, T8 and SS3. No activity was observed in the murine 3T3 or L cell lines or the human HeLa, LS174T, HT29, HOS, SW620 and HUVEC lines, none of which are of melanocytic origin (see Figure 2 and Table 1).

Figure 2 shows the relative activity of tyrosinase and TRP-1 promoters in murine B16.F1 melanoma and NIH 3T3 cells. Cells were transfected with 10 μ g of the appropriate plasmid DNA using the calcium phosphate method. 72-96 hours after the calcium phosphate precipitate had been washed away the cells were analysed for expression of β -galactosidase using the quantitative MUG assay. Data are expressed as mean of triplicate values \pm SD. The data presented are representative of four similar experiments.

In contrast, the SV40 promoter was able to direct expression of the reporter gene to high levels in both cell types.

Table 1

Species	Cell Line	Tissue Type	Expression of:	
			<i>Tyr-β-Gal</i>	<i>TRP-1-β-Gal</i>
Mouse	Melab	Melanocyte	+	+
	B16	Melanoma	+	+
	1735P	Melanoma	+	+
	1735 C19	Melanoma	+	+
	NIH3T3	Fibroblast	-	-
	L cells	Fibroblast	-	-
	AKR	T cell leukaemia	-	-
	Colo 26	Colon	-	-
Rat	Gli C	Glioma	-	-
Hamster	BHK-21	Kidney	-	-
Human	SK23	Melanoma	+	-
	HMB2	Melanoma	+	+
	5S3	Melanoma	+	+
	Mel 8	Melanoma	+	+
	Mel 17	Melanoma	+	+
	TXM13	Melanoma	+	+
	T8	Melanoma	+	+
	A375M	Melanoma	+	+
	VUP	Ocular Melanoma	-	-
	DX3	Melanoma	-	-
	HeLa	Cervical carcinoma	-	-
	HOS	Osteosarcoma	-	-
	HT29	Colorectal carcinoma	-	-
	SW620	Colorectal carcinoma	-	-
	LS174T	Colorectal carcinoma	-	-
	HUVEC	Endothelium	-	-

Footnote to Table 1. Cell type specificity of expression of β -galactosidase from Tyrosinase and TRP-1 promoters. Each cell line indicated was transfected with 10 μ g of plasmid DNA of Tyr- β -Gal 1 and 2, TRP-1- β -Gal 1 and 2. pNASS- β and SV40- β -Gal were used in each case as a negative and positive control for transfection. Expression of β -galactosidase was scored as positive (+) if several cells stained blue 96 hours after transfection; a cell line was scored as negative (-) if no blue cells were observed after transfection and if the quantitative MUG assay showed no expression above background levels (transfection with pNASS-

β).

These results confirm and extend the reports of other groups showing excellent tissue specificity of gene expression in melanocytic cells of either murine or human origin when the 5' promoter regions of either the tyrosinase or TRP-1 gene are utilised.

Example 2: Materials and methods pertaining to the other Examples.

10 Construction of Expression Plasmids and Retroviral Vectors. Subcloning was carried out via standard recombination DNA techniques (Sambrook *et al* (1989) *Molecular cloning, a laboratory manual*, Cold Spring Harbor Laboratory Press, NY, USA). Restriction endonuclease enzymes were supplied by Northumbria Biologicals (NBL, Cramlington, UK) and Taq polymerase was supplied by Stratech (Luton, UK). Oligonucleotides, synthesised on an Applied Biosystems 380B and purified by denaturing acrylamide electrophoresis, were provided by the Oligonucleotide Synthesis Laboratory, ICRF Clare Hall, South Mimms, UK. Polymerase chain reaction (PCR) amplification of DNA fragments was carried out on a Techne PHC-2 Thermocycler and reaction mixes were prepared in a hood separate from normal areas of DNA handling. Amplified DNA sequences were subcloned into the PCR II vector (Invitrogen; British Biotechnology Products Ltd, Oxford, UK) and their identities were confirmed by restriction endonuclease mapping. The correct fragments were then shuttled from PCR II into the appropriate expression plasmid.

Cell Culture. All cell lines used in this study were checked routinely and found to be free of mycoplasma infection. Apart from Melab cells which were cultured in medium supplemented as described previously (Burrows

et al (1991) *Cancer Res.* 51, 4768-4775) the lines were grown in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum and 4 mM L-glutamine. HUVEC (Human umbilical vein endothelial cells) were maintained in Medium 199 (Gibco-Biocult Ltd, Paisley, Scotland) supplemented with Earle's salts, 20% (v/v) fetal calf serum, endothelial cell growth supplement (0.12 mg/ml) 0.09 mg/ml heparin and glutamine. Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10% CO₂.

10 DNA Transfection. 10⁶ adherent cells were transfected with 10 µg of plasmid DNA by calcium phosphate co-precipitation using the Profection method (Promega, Madison, WI) according to the manufacturer's instructions. 24 hours after the application of the precipitate to the tissue culture medium, cells were washed three times in serum-free medium and
15 incubated in normal medium for 72-96 hours when they were stained for β-galactosidase expression.

Intra-Tumoral Injection of DNA. 1-1.5 x 10⁵ tumour cells of either the B16 F1 murine melanoma or the Colo 26 colon carcinoma were injected
20 subcutaneously in 100 µl inoculum volumes into the flank region of syngeneic mice (C57 for B16 F1, Balb/C for Colo 26). Ten days later the animals were anaesthetised by halothane inhalation (ICI Pharmaceuticals, Macclesfield, UK), the tumours, approximately 4 mm in diameter, were located by palpation and injected with 1 µg DNA in 100 µl volumes of
25 either PBS or as calcium phosphate precipitates via a 27-gauge needle.

Quantitative Assay for β-Galactosidase Expression. Transfected cells were assayed for enzyme activity by the technique of MacGregor *et al* (1991) *Methods in Molecular Biology* 7, 217-235 (Ed., E.J. Murray)
30 Humana Press Inc, Clifton, NJ, USA. Briefly cells were resuspended in

Z buffer (60 mM $\text{Na}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10 mM KCl, 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) at 10^7 cells per ml. 105 μl of this cell suspension were dispensed per well of a microtiter plate and 15 μl of 1% Triton X-100 were added to each well to give a final concentration of 0.1%. After 5 10 minutes at room temperature, 30 μl of 3 mM methylumbelliferyl- β -D-galactoside (MUG) (Sigma, Poole, UK) in Z buffer were added to each well and the reaction was allowed to proceed for 90 minutes at 37°C. 75 μl of 300 mM glycine, 15 mM EDTA, pH 11.2 were added to stop the reaction. Fluorescence was measured on a microtiter dish fluorescence 10 reader (excitation at 350 nm and emission read at 450 nm).

Cells expressing β -galactosidase convert the MUG substrate, a non-fluorescent galactoside analogue, to the fluorescent molecule 4-methylumbelliferone.

15

Histochemical Detection of β -Galactosidase-expressing Cells. 72-96 hours following DNA transfection, adherent cells were washed once in phosphate buffered saline (PBS) and fixed for 10 minutes at 4°C with 3.8% formaldehyde in PBS. The fixative was removed by three washes 20 with PBS and the cells were then incubated with X-gal solution [5-bromo-4-chloro-3-indoyl- β -galactopyranoside (Sigma) at 40 mg/ml in dimethylformamide was diluted to 1 mg/ml in 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$; 5 mM $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$; 2 mM MgCl_2 ; 0.01% sodium deoxycholate; 0.2% NP40. All solutions were prepared using glass] at 37°C for at least 4 25 hours according to published techniques (Bondi *et al* (1982) *Histochem.* 76, 153-158). After staining the X-gal solution was removed, the cells were washed three times in PBS and the cells were inspected under a light microscope. Cells expressing the β -galactosidase gene hydrolyse the chromogenic substrate X-gal to give the blue dye bromochloroindole. 30 Control untransfected cells also were stained to assess the background

endogenous β -gal staining.

Detecting of β -Galactosidase-expressing Tumour Cells. 2, 4, 6 or 10 days after injection of DNA into the tumours, animals were killed by CO₂ inhalation, their tumours were excised, minced to 1 mm cubes with scalpels and pushed through a stainless steel sieve with a 5 ml syringe plunger, into culture medium. An aliquot of the resulting cell suspension was spun onto a glass microscope slide using a cytospin centrifuge. Slides were air-dried then fixed for 5 minutes in 3.8% formaldehyde in PBS.

10 The cells were rinsed in PBS and incubated overnight in X-gal stain before being inspected under a light microscope for the presence of blue cells.

Generation of Recombinant Retrovirus Stocks. The AM 12 packaging cell line (Markowitz *et al* (1988) *Virol.* 167, 400-406) containing the packaging constructs for Moloney Leukaemia Virus was transfected with 10 μ g of retroviral plasmid DNA using the calcium phosphate co-precipitation method. 48 hours following transfection the cells were split into puromycin (Sigma) selection medium (1 μ g/ml) and surviving colonies were selected and pooled two weeks later. Virus was harvested from these producer cells by exposing fresh medium to 5 x 10⁶ cells on a 90 mm plate and harvesting the medium 16 hours later. The medium was filtered through a 0.45 μ m filter (Nalge (UK) Ltd, Rotherwas, England) to remove cell debris and was then used to infect target cells. The target cells were split 24 hours earlier to a density of 10⁵ cells per 90 mm plate. Polybrene (Aldrich, Gillingham, Dorset) was added to the viral supernatant to 4 μ g/ml to enhance virus-cell surface interactions and the target cells were exposed to 1 ml of viral supernatant for 2.5 hours at 37°C. 8 ml of normal growth medium were added to the plate and the infected cells were grown for a further 72-96 hours before being stained for expression of β -galactosidase.

30

Example 3: Preparation of tyrosinase promoter- or TRP-1 promoter-driven expression vectors containing cytokine cDNA's.

The pBCMGNeo-mIL-2 vector was provided by Dr P. Frost, University of Texas, Houston and is described in *Eur. J. Immunol.* 18, 97-194 (1988), although other vectors are suitable. This vector had been used to transfect B16 melanoma cells (a non-cell-type-specific approach) and IL-2 producing cells had been selected (Fearon *et al* (1990) *Cell* 60, 397-403). The HCMV promoter of this vector was removed by *Xba*1-*Sal*1 digestion and replaced with the 1.4kb *Xba*1-*Sal*1 fragment of TRP-1 5' sequences or the 780bp tyrosinase 5' sequence fragment generated by Pair 3 oligonucleotides. These constructs were transfected into murine B16 melanoma cells or 3T3 fibroblasts. For the TRP-1 - IL2 construct a total of 60 puromycin-resistant clones were isolated and screened by ELISA for IL-2 production (Genzyme Ltd). Clones were characterised as high (≥ 960 pg/ml), intermediate (150-960 pg/ml) and low (≤ 150 pg/ml) expressers. Of the 60 clones, 13 clones were found to be producing and secreting measurable quantities of IL-2 while ten clones of 3T3 cells and four pooled bulk populations of 3T3 did not contain any cells expressing detectable amounts of IL-2 activity. These results show that the tissue-specific promoter, TRP-1, is able to drive expression of a cytokine cDNA in an appropriate cell type. Repeated analysis over a 6-8 week culture period showed that the observed phenotype is stable.

Alternatively, the IL-2 coding sequence can be incorporated into a tyrosinase promoter vector as follows:

The murine IL-2 cDNA is PCR amplified from pBCMGNeo mIL-2 using the primers GCGGCCGCGCATGTACAGCATGCAGCTCGCA (SEQ ID No 19) and GCGGCCGCTAAATAAATAGAGAGCCTTATG (SEQ ID

No 20).

The PCR fragment is cloned into the vector PCRII (available from Invitrogen) and then excised from the PCRII vector using *NotI* digestion.

- 5 The *NotI* fragment is cloned into the *NotI* site of Tyr- β -Gal-1 (described in Example 1) in place of the β -galactosidase gene. This produces Tyr IL-2 with a 2494 bp promoter from the tyrosinase gene driving expression of IL-2.

- 10 B16 clones have been injected into groups of syngeneic C57 mice. To date only the cell clone selected for drug resistance, ie lacking IL-2 expression, is forming progressively growing tumours in these animals. The IL-2 secreting B16 cells are not forming palpable tumours and, if they do develop, are clearly growing at a slower rate *in vivo*.

15

In addition to the cells secreting IL-2, IL-2 expression is assessed using RT-PCR wherein RNA is isolated, primers such as oligo dT used to prime synthesis of cDNA from the mRNA using reverse transcriptase and the level of IL-2 RNA estimated by amplifying with IL-2-specific
20 oligonucleotides.

20

- We have placed cDNA for IL-4 (bought from British Biotechnology Ltd) downstream of both promoter sequence but the construct may utilise any cytokine gene (eg GM-CSF, TNF, IFN), be combined with the HSV tk
25 gene for ganciclovir selection, or may utilise cDNAs encoding for genes which might stimulate the immune response (eg MHC antigens, MAGE (melanoma antigens) etc). This procedure allows targeted expression of the requisite gene to the cell type of interest, ie melanocyte-derived cells. Replacement of the tyrosinase or TRP-1 promoter sequences with
30 sequences which are expressed by other tumour types in a specific fashion

(eg 5' promoter sequences of the CEA gene for colorectal tumours, 5' sequences of prostate secreted antigen for prostatic tumours) permits targeted expression of similar genes to other tumour types.

5 **Example 4: Introduction of tissue specific promoter-driven genes into target cells *in vivo*.**

There are two main routes of delivery:-

- 10 1) Retroviral delivery
 2) Direct delivery

Incorporation into a Retroviral Vector. The ability of the melanocyte-specific promoters to function after delivery via a retroviral vector was
15 examined because retroviral-mediated gene delivery is a promising route for delivery of gene therapy *in vivo* (Miller (1992) *Nature* 357, 455-460). The retroviral vector pBabe Tyr- β -Gal was constructed from the pBabe Puro vector (Morgenstern & Land (1990) *Nucl. Acids Res.* 18, 3587-3596) (Figure 3). Here β -galactosidase is expressed from the 769 bp tyrosinase
20 promoter fragment of Tyr- β -Gal 2 inserted into pBabe Puro in the opposite orientation to the direction of expression of the viral mRNA driven from the Moloney Leukaemia Virus (MLV) Long Terminal Repeat (LTR).

25 Following transfection of the vector into the AM12 amphotropic packaging cell line, recombinant retroviral particles were used to infect either B16 or NIH 3T3 cells. 72-96 hours following infection, expression of the β -galactosidase gene was observed preferentially in B16 cells relative to the NIH 3T3 target cells by both histochemical and fluorimetric assays.

These results demonstrate that the tyrosinase and TRP-1 promoters can confer tissue specificity of expression upon an heterologous gene in both human and murine melanocyte-derived cell lines when delivered in the context of a retroviral vector.

5

Experiments on route 2 have yielded interesting results. Syngeneic C57/BL mice were injected s.c. in the flank region with 1×10^5 B16 cells and the animals were monitored until a tumour of approximately 0.4×0.4 cm had developed. Similar Colo tumours were established in Balb-C mice. At this time a single injection of $1.0 \mu\text{g}$ of the tyrosinase promoter/pNASS DNA was inoculated in $100 \mu\text{l}$ volumes directly into the centre of the tumour either as 'naked' DNA or as calcium phosphate-coprecipitated material. Similarly, pNASS- β and TRP- β -Gal-2 DNA was inoculated. At varying times thereafter, for example at 2, 4, 6 or 10 days, mice were killed, and the tumours were removed and snap-frozen. Cryostat sections of these tumours were stained for β -galactosidase activity. Protein expression, manifest by the detection of bright blue cells, was clearly apparent in the majority of the injected tumours. The Tyr- β -Gal 2 construct caused the gradual accumulation of positive blue cells in the injected B16 tumours over the ten day period of examination; whereas the same construct injected into the non-melanocytic Colo 26 tumours produced no blue staining. Similar results were obtained in three independent replicate experiments and from these it was apparent that:- (1) the promoterless, control pNASS β construct produced no blue cells in either Colo 26 or B16 tumours; (2) there was a gradual increase in the proportion of blue cells in the positive groups over the 10 day period of examination (10 days was the last time-point examined because of increasing tumour burden) up to an estimated 10-15% of cells (3) no qualitative or quantitative difference was obvious between the tyrosinase or TRP-1 promoter elements or between material injected as naked DNA

or as a CaPO_4 -precipitate. Frozen sections of B16 tumours stained 10 days after DNA injection showed similar results. Interestingly the only blue-staining tissue, apart from the neoplastic cells, was confined to the base of the hair follicles and thus, presumably, indicated transduction of
5 normal melanocytes.

These results show that direct gene transfer may be accomplished by intra-tumoural injections. Morphological assessment of the sections indicated that the blue cells were restricted to areas occupied by neoplastic tissue,
10 which is presumed to reflect the tissue specificity conferred by the 5' tyrosinase or TRP-1 gene sequence.

These experiments suggest that direct injections permit good levels of expression of introduced genes. The activity produced may be altered by
15 modification of the introduced DNA (eg incorporation in liposomes, use of different precipitating material, variation in route of delivery). Taken in combination our results indicate that placing therapeutic genes under control of tissue-specific promoter regions may restrict expression to cells of a specific lineage. This could be important both for safety/specificity
20 purposes and would permit the refinement of what otherwise may be a fairly non-specific event. The utilisation of a cytokine gene has been shown to induce modifications in subsequent tumour behaviour. Direct delivery of DNA via an intratumoural injection has been shown to produce high levels of expression of the introduced gene suggesting that such
25 promoter-restricted expression may be further limited to the target cells by the simple expedient of targeting inoculation. The use of genes encoding for proteins capable of eliciting a subsequent systemic response may permit this method to be used for disseminated, rather than localised, neoplastic disease.

Example 5: c-erbB-2 promoter and reporter enzyme

Reporter enzyme gene. The bacterial chloramphenicol acetyl transferase (CAT) gene was obtained from Promega as the "pCAT-basic" vector.

5

The CAT reporter system is designed to allow sensitive and rapid testing for eukaryotic transcriptional regulatory sequences. This reporter system relies on the linkage of genomic DNA fragments containing putative regulatory sequences to the chloramphenicol acetyltransferase (CAT) reporter gene. Transcriptional effects upon the CAT reporter gene are detected after transfection into cultured cells. Since CAT is a bacterial gene, levels of CAT enzyme activity in crude cell extracts can be quickly and easily assayed with little or no background from endogenous cellular gene activity. The pCAT-Basic plasmid lacks eukaryotic promoter and enhancer sequences. This allows the researcher maximum flexibility in cloning any putative regulatory sequences into the convenient multiple cloning sites. Expression of CAT activity in cells transfected with this plasmid is dependent on insertion of a functional promoter upstream from the CAT gene. Enhancer elements can be inserted upstream from the promoter or at the *Bam*HI site downstream from the CAT gene. Sequences to be tested for transcriptional activity can be cloned into the following unique sites located immediately upstream from the CAT gene: *Xba*I, *Acc*I, *Sal*I, *Pst*I, *Sph*I and *Hind*III. Enhancer elements can be cloned separately into the *Bam*HI site downstream from the CAT transcriptional unit. The vector also contains the gene for ampicillin resistance.

10

15

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Promoter. The human c-erbB-2 promoter has been cloned to -500 by two groups (Ishi *et al* (1987) *Proc Natl Acad Sci USA* 84, 4374-4378; Tal *et al* (1987) *Mol Cell Biol* 7, 2597-2601) and to -1500 by a third group

(Hudson *et al* (1990a) *J Biol Chem* 265, 4389-4393). We have taken oligonucleotides to 30b regions around +40 and -500 and, using PCR against human genomic DNA, recovered a 540bp fragment representing the *c-erbB-2* proximal promoter. Using oligos to -1000 and -500 we then

5 "PCRed" out a further 500bp representing the *c-erbB-2* distal promoter. The two promoter regions were fused at the *SmaI* site at -500 and the full promoter cloned upstream of the CAT gene to generate a reporter plasmid for assaying *c-erbB-2* promoter activity in cell lines *in vitro*. Further constructs were made by either deleting 5' regions of the promoter using

10 convenient restriction enzyme sites, or using PCR technology, to generate a series of promoter deletion mutants linked to CAT 3' end always +40; 5' ends as follows: -1000, -500, -400, -300, -213, -177, -100; (Figure 1).

15 **Construction of *c-erbB-2* plasmid.** The *c-erbB-2* promoter was incorporated in the pCAT-basic plasmid to give the plasmid shown in Figure 1 by digesting the plasmid with *XbaI* and then filling the ends with Klenow fragment to create a blunt-ended vector suitable for cloning the blunt-ended PCR products.

20

The CAT activity from the various promoter constructs was compared to baseline activity from the promoterless CAT parent plasmid by calcium phosphate mediated DNA transfection into a number of different breast cell lines. Immortalised normal and tumour lines which have low

25 endogenous *c-erbB-2* expression showed little activity of the *c-erbB-2* promoter, ie all the reporter constructs containing *c-erbB-2* sequences generated no more CAT activity than the promoterless control plasmid. This result makes it unlikely that *c-erbB-2* expression is actively repressed in these cell lines (by a tumour suppressor-like activity).

30

Example 6: Promoter region of the carcinoembryonic antigen gene

The CEA gene is cloned using standard methods as described by Schrewe
et al (1990) *Mol. Cell. Biol.* 10, 2738-2748 and sequenced using the
5 dideoxy chain termination method of Sanger *et al* (1980) *J. Mol. Biol.*
143, 161-178.

To define the actual portion of the 5' untranslated region which is required
for the promoter activity of the CEA gene, we carried out functional tests
10 by placing restriction endonuclease fragments of various lengths from the
putative promoter regions of both genes upstream of the CAT reporter
gene and assaying for CAT activity in a transient transfection assay in two
different human cell lines. For this purpose, we chose the CEA-producing
adenocarcinoma cell line SW403 and, as a negative control, the HeLa cell
15 line. The CEA promoter constructs showed an enhanced expression of the
CAT gene in SW403 cells, which was nine times greater than in HeLa
cells, when the shortest construct was used. It appears that *cis* regulatory
sequences, which are responsible for this enhancement, along with a
functional transcription initiator, are both present within the first 424
20 nucleotides upstream of the translational start. It is also interesting that
longer CEA constructs are approximately 50% less active in HeLa cells
than is the shortest construct. A possible explanation for this phenomenon
is that a silencer region could exist between nucleotides
-424 and -832 upstream from the translational start, which reduces the
25 activities in both cell lines through interaction with common *trans*-acting
regulatory factors. Such silencer sequences have indeed been described
for other genes.

Thus, the promoter of the CEA gene is useful for expressing cytokines,
30 according to the methods of the invention, in colon tumours.

As found here for CEA, a number of other eucaryotic genes have also been reported which do not contain obvious TATA boxes. The promoters of such genes can be divided into two classes. The members of the first class are G+C rich and are found primarily in housekeeping genes.

5 These promoters usually contain several transcription initiation sites spread over a fairly large region, as well as potential binding sites for Spl. The members of the second class are not G+C rich, are not constitutively active, but are regulated during differentiation or development and initiate transcription at only one or a few tightly clustered start sites. Included in

10 this class are a number of genes that are regulated during mammalian immunodifferentiation, eg the T-cell receptor β -chain genes and the V_{preB} gene, as well as some *Drosophila* homeotic genes. The CEA gene shows a closer resemblance to this latter group, because its promoter is not obviously G+C rich, it contains no identifiable Spl-binding sites, it

15 reveals only a few tightly clustered start sites, and, most importantly, it is not constitutively expressed.

Figure 6 shows the nucleotide sequence from the promoter region of CEA compared with the promoter region of the non-specific cross-reacting

20 antigen gene (NCA) and the CGM1 gene. The numbers indicate the distance in nucleotides from the initiation codon for each gene. Gaps have been introduced to allow optimal alignment. Identical nucleotides are indicated by dots. The cluster of transcriptional start sites determined for CEA and NCA by S1 nuclease assays are indicated by arrows.

25

Example 7: Promoter region of the prostate-specific antigen gene

The PSA gene is cloned using standard methods as described by Riegman *et al* (1989) *Biochem. Biophys. Res. Comm.* 159, 95-102 and Lundwall

30 (1989) *Biochem. Biophys. Res. Comm.* 161, 1151-1159 and sequenced

using the dideoxy chain termination method of Sanger *et al* (1980) *J. Mol. Biol.* 143, 161-178.

5 The sequence of the promoter region of PSA gene, compared to that of the hGK-1 gene, is shown in Figure 7. Dots represent identical nucleotides. Putative transcriptional regulatory elements are boxed.

PSA is expressed at a high level in the prostate; hGK-1, a human kallikrein-like gene, is expressed at lower level in the prostate.

10

The differences in nucleotide sequence between the PSA and hGK-1 promoters are probably important determinants in prostate-specific gene expression.

15 Thus, the promoter of the PSA gene is useful for expressing cytokines, according to the method of the invention, in prostate tumours.

Example 8: Promoter region of the MUC1 gene

20 The mucin gene, MUC1, is selectively expressed in breast and pancreatic cell lines but not in non-epithelial cell lines. The promoter region for this gene may be obtained by the methods taught in WO 91/09867.

25 The 5' sequences flanking the human MUC1 gene are analyzed for their ability to direct expression of a reporter gene (the chloramphenical transferase gene, CAT) in cell lines which normally express or do not express the MUC1 gene. A construct containing 2.9 kb of MUC1 5' flanking sequence shows expression of CAT in breast and pancreatic cell lines but not in the non-epithelial cell lines HT 1080, SK23 and HTB96.

30 Deletion analysis shows that maximum expression was obtained in ZR-75

- (breast cancer line) and HPAP (pancreatic cancer line) with only 743 bp of 5' flanking sequence. Sequences within 1.6 kb of the transcriptional start site showed enhancing activity in a vector carrying an enhancerless SV40 promoter. Analysis of proximal 5' sequences in a promoterless
- 5 CAT vector carrying the SV40 enhancer shows that sequences between -60 and -150 were crucial for tissue specific expression. An Spl site at -99/-90 and an E-box (E-MUC1) at -84/-64 in this region are shown by mutational analysis to play a role in the regulation of transcription. Gel
- 10 shift analysis with oligonucleotides and nuclear extracts of ZR-75 showed protein binding to both of these sites. Spl binding activity is similar in ZR-75 and HT1080 cells whereas binding of factors to the E-MUC1 oligonucleotide reveals quantitative and qualitative differences between epithelial and non-epithelial cells.
- 15 Thus, the promoter of the MUC1 gene is useful for expressing cytokines, according to the method of the invention, in pancreatic and breast tumours.

Example 9: Treatment of patients

20

1. **Patient selection**
 - a) Patients with metastatic malignant melanoma with good performance data (WHO Grade zero 1 or 2) with a life expectancy of at least three months, normal renal and liver function and haematology,
 - 25 normal bilirubin and no evidence of cerebral secondaries are selected.
 - b) Written consent is obtained.
 - c) Patients need not have received prior chemotherapy because of the low activity, toxicity and immunosuppression of such treatments. They can be administered after the gene therapy is completed, if indicated.
 - 30 d) Diagnosis of metastasis is confirmed by fine needle aspiration

cytology.

2. Administration of constructs

- 5 a) The constructs used are composed of a 769 bp fragment or a 2.5 kb fragment of the 5' flanking sequence of the murine tyrosinase gene driving the human IL-2 gene within the promoterless mammalian expression vector pNASS β (Clontech, Ca, USA). The decision to use the murine promoter sequence is based upon our demonstration that this sequence works well in human cells. Initial purification of the bulk grown
- 10 plasmids DNA is achieved using QIAGEN-tips for plasmid purification (this is an anion exchange resin). The bacterial cells used as recipients for the plasmid constructs are the *E. coli* strain JM109. Verification of plasmid purity is by agarose gel electrophoresis. It is prepared to the same pyrogen free standards as monoclonal antibodies which are given in
- 15 much higher amounts. It is administered in sterile saline.
- b) All injections are given by a qualified medical practitioner with MRCP or equivalent and training in medical oncology. A 27 gauge needle is used and local anaesthetic administered first.
- c) Patients are admitted for 24 hours following the injection and will
- 20 be seen at three days and one week and thereafter weekly for one month and then monthly. The injection site is carefully examined and analgesia given as necessary.

3. Studies on initial needle aspirate for diagnostic purposes

- 25 a) immunocytochemistry for melanoma cells and assessment of cell cycle distribution.
- b) PCR to assess cytokine expression - IL-2, interferon- γ and TNF α .

4. Dosage schedule
 tyrosinase/IL-2

	Dose	Biopsy
	Cohort 1 100 μ g DNA/200 μ l	1 week
5	2 100 μ g DNA/200 μ l	2 weeks

5. Studies of excisional biopsy after construct injection

- a) immunochemistry for melanoma cells.
- b) genomic PCR to assess the construct.
- 10 c) staining for lymphocyte sub-populations and dendritic cells, PCR for IL-2 interferon- γ and TNF α . *In situ* hybridisation for the same cytokines.
- d) assessment of cytotoxic T cell response to autologous melanoma cells. Cells obtained from the biopsies will be used in chromium release
- 15 assays, as well as peripheral T cells.

6. Studies on stored DNA preparations

- a) In order to verify that the prepared DNA has not been degraded, routine examination of an aliquot of the injected material by agarose gel
- 20 electrophoresis should be carried out.

Assessment of results

The effectiveness of this approach is assessed by three criteria.

25 **1) Assessment of IL-2 expression by RTPCR *in situ* hybridisation and immunochemistry**

A similar level of expression within 10-15% of tumour cells is found.

2) Assessment of local immune response by immunocytochemistry

Lymphocyte subpopulations and dendritic cells are stained to assess subtypes of cells present after the injections.

5

3) Assessment of cytotoxic T cell responses

There is 1-2 weeks of local IL-2 production.

10 There is a demonstration of a positive T cell response.

Genes that can be expressed include cytokines such as TNF α , GM-CSF, IL-4, interferon- γ or the proteins involved in T cell antigen recognition like class 1 molecules or B7.

15

Safety

Considering the life expectancy of these patients who already have metastatic cancer, the risks of insertion of genetic material into the somatic
20 cells of the body would appear to be minimal. Clearly there may be events resulting from positional integration into the genome, eg insertional mutagenesis, inactivation or enhancement of expression, which could theoretically be deleterious. However, these have not manifested themselves in over 200 injections into recipient mice and their importance
25 appears to be more theoretical than practical. Moreover, should adverse immunological reactions occur, they are unlikely to be beyond control with a range of immunosuppressive agents. Again, the short life expectancy of these patients makes long term undesirable sequelae an unlikely event. The risks of chemotherapy with marrow suppression,
30 allergic reactions, Budd-Chiari syndrome and infection would all seem to

pose much greater clinical problems than the local injection of DNA.

Example 10: Co-injection of IL-2 expressing and B7-expressing DNA constructs into a melanoma

5

A TRP-1-B7 construct is made using PCR, the sequence information in the sequence listing and a DNA vector such that expression of the B7 coding sequence is driven by the TRP-1 promoter.

- 10 The TRP-1-B7 construct and the TRP-1-IL-2 construct of Example 3 are prepared in sterile, pyrogen free water. 100 μ g of each DNA construct in 200 μ l of water is injected into the melanoma at weekly intervals until the tumour regresses.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Imperial Cancer Research Technology Ltd
 (B) STREET: Sardinia House, Sardinia Street
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 (I) TELEX: 265107 ICRF G

(ii) TITLE OF INVENTION: Tumour therapy

(iii) NUMBER OF SEQUENCES: 22

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3281 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAGCTCCTCA CACGGACTCT GTCAGCTCCT CCCTGCAGCC TATCGGCCGC CCACCTGAGG	60
CTTGTCGGCC GCCCACTTGA GGCCTGTCGG CTGCCCTCTG CAGGCAGCTC CTGTCCCCTA	120
CACCCCTCC TTCCCGGGC TCAGCTGAAA GGGCGTCTCC CAGGGCAGCT CCCTGTGATC	180
TCCAGGACAG CTCAGTCTCT CACAGGCTCC GACGCCCCCT ATGCTGTCAC CTCACAGCCC	240
TGTCATTACC ATTAACCTCT CAGTCCCATG AAGTTCATG AGCGCCTGTC TCCCGGTAC	300
AGGAAACTC TGTGACAGGG ACCACGTCTG TCCTGCTCTC TGTGGAATCC CAGGGCCCAG	360
CCAGTGCCTG ACACGGAACA GATGCTCCAT AAATACTGGT TAAATGTGTG GGAGATCTCT	420
AAAAAGAAAC ATATCACCTC CGTGTGGCCC CCAGCAGTCA GAGTCTGTTC CATGTGGACA	480
CAGGGGCACT GGCACCAGCA TGGGAGGAGG CCAGCAAGTG CCCGCGGCTG CCCCAGGAAT	540
GAGGCCTCAA CCCCAGAGC TTCAGAAGGG AGGACAGAGG CCTGCAGGGA ATAGATCTCT	600
CGGCCTGACC CTGCAGCCTA ATCCTGAGTT CAGGGTCAGC TCACACCACG TCGACCCTGG	660

TCAGCATCCC	TAGGGCAGTT	CCAGACAAGG	CCGGAGGTCT	CCTCTTGCCC	TCCAGGGGGT	720
GACATTGCAC	ACAGACATCA	CTCAGGAAAC	GGATTCCCCT	GGACAGGAAC	CTGGCTTTGC	780
TAAGGAAGTG	GAGGTGGAGC	CTGTTTCCA	TCCCTTGCTC	CAACAGACCC	TTCTGATCTC	840
TCCCACATAC	CTGCTCTGTT	CCTTTCGGG	TCCTCTGAGG	ACCTGTTCTG	CCAGGGGTCC	900
CTGTGCAACT	CCAGACTCCC	TCCTGGTACC	ACCATGGGGA	AGGTGGGGTG	ATCACAGGAC	960
AGTCAGCCTC	GCAGAGACAG	AGACCACCCA	GGACTGTCAG	GGAGAACATG	GACAGGCCCT	1020
GAGCCGCAGC	TCAGCCAACA	GACACGGAGA	GGGAGGGTCC	CCCTGGAGCC	TTCCCCAAGG	1080
ACAGCAGAGC	CCAGAGTCAC	CCACCTCCCCT	CCACCACAGT	CCTCTCTTTC	CAGGACACAC	1140
AAGACACCTC	CCCCTCCACA	TGCAGGATCT	GGGGACTCCT	GAGACCTCTG	GGCCTGGGTC	1200
TCCATCCCTG	GGTCAGTGGC	GGGGTTGGTG	GTACTGGAGA	CAGAGGGCTG	GTCCCTCCCC	1260
AGCCACCACC	CAGTGAGCCT	TTTTCTAGCC	CCCAGAGCCA	CCTCTGTCAC	CTTCCTGTTG	1320
GGCATCATCC	CACCTTCCCA	GAGCCCTGGA	GAGCATGGGG	AGACCCGGGA	CCTGCTGGGT	1380
TTCTCTGTCA	CAAAGGAAAA	TAATCCCCCT	GGTGTGACAG	ACCCAAGGAC	AGAACACAGC	1440
AGAGGTGAGC	ACTGGGGAAA	GACAGGTTGT	CCACAGGGGA	TGGGGGTCCA	TCCACCTTGC	1500
CGAAAAGATT	TGTCTGAGGA	ACTGAAAATA	GAAGGGAAAA	AAGAGGAGGG	ACAAAAGAGG	1560
CAGAAATGAG	AGGGGAGGGG	ACAGAGGACA	CCTGAATAAA	GACCACACCC	ATGACCCACG	1620
TGATGCTGAG	AAGTACTCCT	GCCCTAGGAA	GAGACTCAGG	GCAGAGGGAG	GAAGGACAGC	1680
AGACCAGACA	GTCACAGCAG	CCTTGACAAA	ACGTTCTCTG	AACTCAAGCT	CTTCTCCACA	1740
GAGGAGGACA	GAGCAGACAG	CAGAGACCAT	GGAGTCTCCC	TGGGCCCTC	CCCACAGATG	1800
GTGCATCCCC	TGGCAGAGGC	TCCTGCTCAC	AGGTGAAGGG	AGGACAACCC	CTGGGAGAGG	1860
GTGGGAGGAG	GGAGCACAGA	GACTGGCTGG	GGTCTCCTGG	GTAGGACAGG	GCTGTGAGAC	1920
GGACAGAGGG	CTCCTGTTGG	AGCCTGAATA	GGGAAGAGGA	CATCAGAGAG	GGACAGGAGT	1980
CACACCAGAA	AAATCAAATT	GAATGGAAT	TGGAAAGGGG	CAGGAAAACC	TCAAGAGTTC	2040
TATTTTCCTA	GTTAATTGTC	ACTGGCCACT	ACGTTTTTAA	AAATCATAAT	AACTGCATCA	2100
GATGACACTT	TAAATAAAAA	CATAACCAGG	GCATGAAACA	CTGTCCTCAT	CCGCCTACCG	2160
CGGACATTGG	AAAATAAGCC	CCAGGCTGTG	GAGGGCCCTG	GGAACCCTCA	TGAACTCATC	2220
CACAGGAATC	TGCAGCCTGT	CCCAGGCACT	GGGTGCAACC	AAGATCACAC	AAATCCCTGC	2280
CCTCATGAAG	CTCATGCTCT	CATGGGGAGG	AAGACAGACA	TACAAAGAGA	TCTAGAATGT	2340
GAGGTCAGGT	GTTGACAAGA	GCCTGGAGGG	AATAGAGCAG	GGAAAGGTCA	GAAAAGGAAG	2400
ACCCAAGGTC	TCTAGAGGAG	GTGTCAGGGA	AGGGATCTCC	CAAGAATGCC	CTGATGTGAG	2460
CAGGACCTGA	AGGCAATGGG	GAGGGAGCCG	TGAAGACCCC	TGGAAAAGCA	GATTCCACAC	2520
AGGGAAATGC	CAAGGTCGGA	GGTGCTAAGG	AAATAGGAGA	CACACTGCTG	ACCTTGACCT	2580
AGTAGGACAC	ACACACACAC	ACACACACAC	ACTCACTCAC	TCCAGGGCTG	GGGGATGAAG	2640

AGACCTGCTC AGGACCCAGG ACCCCATTTT TCCACCCTAA TGCATAGGTC CCAATATTGA	2700
CCGATGCTCT CTGCTCTCTC CTAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG	2760
CCAAGCTCAC TATTGAATCC ACGCCGTTCA ATGTGCGAGA GGGGAAGGAG GTGCTTCTAC	2820
TTGTCCACAA TCTGCCCCAG CATCTTTTGT GCTACAGCTG GTACAAAGGT GAAAGAGTGG	2880
ATGGCAACCG TCAAATTATA GGATATGTAA TAGGAACTCA ACAAGCTACC CCAGGGCCCCG	2940
CATACAGTGG TCGAGAGATA ATATACCCCA ATGCATCCCT GCTGATCCAG AACATCATCC	3000
AGAATGACAC AGGATTCTAC ACCCTACACG TCATAAAGTC AGATCTTGTG AATGAAGAAG	3060
CAACTGGCCA GTTCCGGGTA TACCGTGAGT GATTCCCCCA TGACCTCTGG GTGTTGGGGG	3120
TCAGTTCTAC TTCCACACA CAGGATTATC AGGCCTGGGC TGTGCTGTGG CCCCCTCTGC	3180
ATTACGAACC ATGTTAGGGT TTGGGCATTT AGTGCAGGAT ACACACAGAA GAGACAAACT	3240
TCAACAGATC AGAATTCCTT TCGGCATCC AGACCCTGCA G	3281

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7130 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATTCCACA TTGTTTGCTG CACGTTGGAT TTTGAAATGC TAGGGAAGTT TGGGAGACTC	60
ATATTTCTGG GCTAGAGGAT CTGTGGACCA CAAGATCTTT TTATGATGAC AGTAGCAATG	120
TATCTGTGGA GCTGGATTCT GGGTTGGGAG TGCAACGAAA AGAATGTACT AAATGCCAAG	180
ACATCTATTT CAGGAGCATG AGGAATAAAA GTTCTAGTTT CTGGTCTCAG AGTGGTGCAG	240
GGATCAGGGA GTCTCACAAT CTCCTGAGTG CTGGTGTCTT AGGGCACACT GGGTCTTGGA	300
GTGCAAAGGA TCTAGGCACG TGAGGCTTTG TATGAAGAAT CGGGGATCGT ACCCAGCCCC	360
TGTTTCTGTT TCATCCTGGG CATGTCTCCT CTGCCTTTGT CCCCTAGATG AAGTCTCCAT	420
GAGCTACAAG GGCCTGGTGC ATCCAGGGTG ATCTAGTAAT TGCAGAACAG CAAGTGCTAG	480
CTCTCCCTCC CTTCCACAG CTCTGGGTGT GGGAGGGGGT TGTCCAGCCT CCAGCAGCAT	540
GGGGAGGGCC TTGGTCAGCC TCTGGGTGCC AGCAGGGCAG GGGCGGAGTC CTGGGGAATG	600
AAGGTTTTAT AGGGCTCCTG GGGGAGGCTC CCCAGCCCCA AGCTTACCAC CTGCACCCGG	660
AGAGCTGTGT CACCATGTGG GTCCCGGTTG TCTTCCTCAC CCTGTCCGTG ACGTGGATTG	720
GTGAGAGGGG CCATGTTTGG GGGGATGCAG GAGAGGGAGC CAGCCCTGAC TGTCAAGCTG	780

AGGCTCTTTC CCCCCCAACC CAGCACCCCA GCCCAGACAG GGAGCTGGGC TCTTTTCTGT	840
CTCTCCCAGC CCCACTTCAA GCCCATACCC CCAGCCCCCTC CATATTGCAA CAGTCCTCAC	900
TCCCACACCA GGTCCCCGCT CCCTCCCACT TACCCAGAA CTTTCTCCCC ATTGCCCAGC	960
CAGCTCCCTG CTCCCAGCTG CTTTACTAAA GGGGAAGTTC CTGGGCATCT CCGTGTCTCT	1020
CTTTGTGGGG CTCAAAACCT CCAAGGACCT CTCTCAATGC CATTGGTTCC TTGGACCGTA	1080
TCACTGGTCC ATCTCCTGAG CCCCTCAATC CTATCACAGT CTAATGACTT TTCCCATTC	1140
GCTGTGAGTG TCCAACCCTA TCCCAGAGAC CTTGATGCTT GGCCTCCCAA TCTTGCCCTA	1200
GGATACCCAG ATGCCAACCA GACACCTCCT TCTTCCTAGC CAGGCTATCT GGCCTGAGAC	1260
AACAAATGGG TCCCTCAGTC TGGCAATGGG ACTCTGAGAA CTCCTCATTG CCTGACTCTT	1320
AGCCCCAGAC TCTTCATTCA GTGGCCCAACA TTTTCCTTAG GAAAAACATG AGCATCCCCA	1380
GCCACAACCTG CCAGCTCTCT GATTCCCCAA ATCTGCATCC TTTTCAAAAC CTAAAAACAA	1440
AAAGAAAAAC AAATAAAACA AAACCAACTC AGACCAGAAC TGTTTTCTCA ACCTGGGACT	1500
TCCTAAACTT TCAAAACCT TCCTCTTCCA GCAACTGAAC CTGGCCATAA GGCACTTATC	1560
CCTGGTTCTT AGCACCCCTT ATCCCCCTAG AATCCACAAC TTGTACCAAG TTTCCCTTCT	1620
CCCAGTCCAA GACCCCAAAT CACCACAAAG GACCCAATCC CCAGACTCAA GATATGGTCT	1680
GGGCGCTGTC TTGTGTCTCC TACCCTGATC CCTGGGTTCA ACTCTGCTCC CAGAGCATGA	1740
AGCCTCTCCA CCAGCACCAG CCACCAACCT GCAAACCTAG GGAAGATTGA CAGAATTCCC	1800
AGCCTTTCCC AGCTCCCCCT GCCCATGTCC CAGGACTCCC AGCCTTGGTT CTCTGCCCCC	1860
GTGTCTTTTC AAACCCACAT CCTAAATCCA TCTCCTATCC GAGTCCCCCA GTTCCCCCTG	1920
TCAACCCTGA TTCCCCTGAT CTAGCACCCC CTCTGCAGGC GCTGCGCCCC TCATCCTGTC	1980
TCGGATTGTG GGAGGCTGGG AGTGCGAGAA GCATTCCCAA CCCTGGCAGG TGCTTGTTGGC	2040
CTCTCGTGGC AGGGCAGTCT GCGGCGGTGT TCTGGTGCAC CCCCAGTGGG TCCTCACAGC	2100
TGCCCCTGTC ATCAGGAAGT GAGTAGGGGC CTGGGGTCTG GGGAGCAGGT GTCTGTGTCC	2160
CAGAGGAATA ACAGCTGGGC ATTTTCCCCA GGATAACCTC TAAGGCCAGC CTTGGGACTG	2220
GGGAGAGAG GGAAGTTCT GGTTCAGGTC ACATGGGGAG GCAGGGTTGG GGCTGGACCA	2280
CCCTCCCCAT GGCTGCCTGG GTCTCCATCT GTGTCCCTCT ATGTCTCTTT GTGTGCTTTT	2340
CATTATGTCT CTTGGTAACT GGCTTCGGTT GTGTCTCTCC GTGTGACTAT TTTGTTCTCT	2400
CTCTCCCTCT CTTCTCTGTC TTCAGTCTCC ATATCTCCCC CTCTCTCTGT CTTTCTCTGG	2460
TCCCTCTCTA GCCAGTGTGT CTCACCCTGT ATCTCTCTGC CAGGCTCTGT CTCTCGGTCT	2520
CTGTCTCACC TGTGCCTTCT CCCTACTGAA CACACGCACG GGATGGGCCT GGGGGGACCC	2580
TGAGAAAAGG AAGGGCTTTG GCTGGGCGCG GTGGCTCACA CCTGTAATCC CAGCACTTTG	2640
GGAGGCCAAG GCAGGTAGAT CACCTGAGGT CAGGAGTTCT AGACCAGCCT GGCCAACTGG	2700
TGAAACCCCA TCTCTACTAA AAATACAAA AATTAGCCAG GCGTGGTGGC GCATGCCTGT	2760

AGTCCCAGCT	ACTCAGGAGG	CTGAGGGAGG	AGAATTGCTT	GAACCTGGGA	GGTTGAGGTT	2820
GCAGTGAGCC	GAGACCGTGC	CACTGCACTC	CAGCCTGGGT	GACAGAGTGA	GACTCCGCCT	2880
CAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AGAAAAGAAA	AGAAAAGAAA	AGGAATCTTT	2940
TATCCCTGAT	GTGTGTGGGT	ATGAGGGTAT	GAGAGGGCCC	CTCTCACTCC	ATTCTTTCTC	3000
CAGGACATCC	CTCCACTCTT	GGGAGACACA	GAGAAGGGCT	GGTTCCAGCT	GGAGCTGGGA	3060
GGGGCAATTG	AGGGAGGAGG	AAGGAGAAGG	GGGAAGGAAA	ACAGGGTATG	GGGGAAAGGA	3120
CCCTGGGGAG	CGAAGTGGAG	GATACAACTT	TGGGCCTGCA	GGCCAGGCTA	CCTACCCACT	3180
TGGAACCCCA	CGCCAAAGCC	GCATCTACAG	CTGAGCCACT	CTGAGGCCTC	CCCTCCCCGG	3240
CGGTCCCCAC	TCAGCTCCAA	AGTCTCTCTC	CCTTTTCTCT	CCCACACTTT	ATCATCCCCC	3300
GGATTCTCTT	CTACTTGGTT	CTCATCTTTC	CTTGACTTTC	CTGCTTCCCT	TTCTCATTCA	3360
TCTGTTTCTC	ACTTTCTGCC	TGGTTTTGTT	CTTCTCTCTC	TCTTTCTCTG	GCCCATGTCT	3420
GTTTCTCTAT	GTTTCTGTCT	TTTCTTTCTC	ATCCTGTGTA	TTTTCGGCTC	ACCTTGTTTG	3480
TCACTGTTCT	CCCCCTCTGC	CTTTCATTCT	CTCTGTCCCT	TTACCCTCTT	CCTTTTTCCC	3540
TGGGTTTCTC	TCAGTTTCTG	TATCTGCCCT	TCACCCCTCT	ACACTGCTGT	TTCCCAACTC	3600
GTGTCTGTA	TTTTTGCCCT	GAAGTGTGTC	TTCCCCAACC	CTGTGTTTTT	CTCACTGTTT	3660
CTTTTCTCTT	TTTGGAGCCT	CCTCCTTGCT	CCTCTGTCCC	TTCTCTCTTT	CCTTATCATC	3720
CTCGCTCCTC	ATTCTGCGT	CTGCTTCCTC	CCCAGCAAAA	GCGTGATCTT	GCTGGGTCCG	3780
CACAGCCTGT	TTCATCCTGA	AGACACAGGC	CAGGTATTTT	AGGTCAGCCA	CAGCTTCCCA	3840
CACCCGCTCT	ACGATATGAG	CCTCCTGAAG	AATCGATTCC	TCAGGCCAGG	TGATGACTCC	3900
AGCCACGACC	TCATGCTGCT	CCGCCTGTCA	GAGCCTGCCG	AGCTCACGGA	TGCTGTGAAG	3960
GTCATGGACC	TGCCCACCCA	GGAGCCAGCA	CTGGGGACCA	CCTGCTACGC	CTCAGGCTGG	4020
GGCAGCATTG	AACCAGAGGA	GTGTACGCCT	GGGCCAGATG	GTGCAGCCGG	GAGCCCAGAT	4080
GCCTGGGTCT	GAGGGAGGAG	GGGACAGGAC	TCCTGGGTCT	GAGGGAGGAG	GGCCAAGGAA	4140
CCAGGTGGGG	TCCAGCCAC	AACAGTGTTT	TTGCCTGGCC	CGTAGTCTTG	ACCCCAAAGA	4200
AACITCAGTG	TGTGGACCTC	CATGTTATTT	CCAATGACGT	GTGTGCGCAA	GTTACCCCTC	4260
AGAAGGTGAC	CAAGTTTCATG	CTGTGTGCTG	GACGCTGGAC	AGGGGGCAAA	AGCACCTGCT	4320
CGGTGAGTCA	TCCCTACTCC	CAAGATCTTG	AGGGGAAAGG	TGAGTGGGGA	CCTTAATTCT	4380
GGGCTGGGGT	CTAGAAGCCA	ACAAGGCGTC	TGCCTCCCCC	GCTCCCCAGC	TGTAGCCATG	4440
CCACCTCCCC	GTGTCTCATC	TCATTCCCTC	CTTCCCTCTT	CTTTGACTCC	CTCAAGGCAA	4500
TAGGTTATTG	TTACAGCACA	ACTCATCTGT	TCCTGCGTTC	AGCACACGGT	TACTAGGCAC	4560
CTGCTATGCA	CCCAGCACTG	CCCTAGAGCC	TGGGACATAG	CAGTGAACAG	ACAGAGAGCA	4620
GCCCCCTCCT	TCTGTAGCCC	CCAAGCCAGT	GAGGGGCACA	GGCAGGAACA	GGGACCACAA	4680
CACAGAAAAG	CTGGAGGGTG	TCAGGAGGTG	ATCAGGCTCT	CGGGGAGGGA	GAAGGGGTGG	4740

GGAGTGTGAC TGGGAGGAGA CATCCTGCAG AAGGTGGGAG TGAGCAAACA CCTGCCGCAG	4800
GGGAGGGGAG GGCCCTGCGG CACCTGGGGG AGCAGAGGGA ACAGCATCTG GCCAGGCCTG	4860
GGAGGAGGGG CCTAGAGGGC GTCAGGAGCA GAGAGGAGGT TGCCTGGCTG GAGTGAAGGA	4920
TCGGGGCAGG GTGCGAGAGG GAAGAAAGGA CCCCTCCTGC AGGGCCTCAC CTGGGCCACA	4980
GGAGGACACT GCTTTTCCTC TGAGGAGTCA GGAAGTGTGG ATGGTGCTGG ACAGAAGCAG	5040
GACAGGGCCT GGCTCAGGTG TCCAGAGGCT GCGCTGGCC TCCCTATGGG ATCAGACTGC	5100
AGGGAGGGAG GGCAGCAGGG ATGTGGAGGG AGTGATGATG GGGCTGACCT GGGGGTGGCT	5160
CCAGGCATTG TCCCCACCTG GGCCCTTACC CAGCCTCCCT CACAGGCTCC TGGCCCTCAG	5220
TCTCTCCCCT CCACTCCATT CTCCACCTAC CCACAGTGGG TCATTCTGAT CACCGAACTG	5280
ACCATGCCAG CCCTGCCGAT GGTCTCCAT GGCTCCCTAG TGCCCTGGAG AGGAGGTGTC	5340
TAGTCAGAGA GTAGTCCTGG AAGGTGGCCT CTGTGAGGAG CCACGGGGAC AGCATCCTGC	5400
AGATGGTCCT GGCCCTGTG CACCGACCT GTCTACAAG ACTGTCTCTG TGGACCCTCC	5460
CCTCTGCACA GGAGCTGGAC CCTGAAGTCC CTTCCTTACC GGCCAGGACT GGAGCCCCTA	5520
CCCCTCTGTT GGAATCCCTG CCCACCTTCT TCTGGAAGTC GGCTCTGGAG ACATTTCTCT	5580
CTTCTTCCAA AGCTGGGAAC TGCTATCTGT TATCTGCCTG TCCAGGTCTG AAAGATAGGA	5640
TGCCCCAGGC AGAAACTGGG ACTGACCTAT CTCACTCTCT CCCTGCTTTT ACCCTTAGGG	5700
TGATTCTGGG GGCCCACTTG TCTGTAATGG TGTGCTTCAA GGTATCACGT CATGGGGCAG	5760
TGAACCATGT GCCCTGCCCG AAAGGCCTTC CCTGTACACC AAGGTGGTGC ATTACCGGAA	5820
GTGGATCAAG GACACCATCG TGGCCAACCC CTGAGCACCC CTATCAACTC CCTATTGTAG	5880
TAAACTTGGA ACCTTGGAAG TGACCAGGCC AAGACTCAAG CCTCCCCAGT TCTACTGACC	5940
TTTGTCTTA GGTGTGAGGT CCAGGGTTGC TAGGAAAAGA AATCAGCAGA CACAGGTGTA	6000
GACCAGAGTG TTTCTTAAAT GGTGTAATTT TGTCTCTCT GTGTCTGGG GAATACTGGC	6060
CATGCCTGGA GACATATCAC TCAATTTCTC TGAGGACACA GATAGGATGG GGTGTCTGTG	6120
TTATTTGTGG GATACAGAGA TGAAAGAGGG GTGGGATCCA CACTGAGAGA GTGGAGAGTG	6180
ACATGTGCTG GACACTGTCC ATGAAGCACT GAGCAGAAGC TGGAGGCACA ACGCACCAGA	6240
CACTCACAGC AAGGATGGAG CTGAAAACAT AACCCACTCT GTCCTGGAGG CACTGGGAAG	6300
CCTAGAGAAG GCTGTGAGCC AAGGAGGGAG GGTCTTCCTT TGGCATGGGA TGGGGATGAA	6360
GTAAGGAGAG GGAAGTGGACC CCCTGGAAGC TGATTCACTA TGGGGGGAGG TGTATTGAAG	6420
TCCTCCAGAC AACCCTCAGA TTTGATGATT TCCTAGTAGA ACTCACAGAA ATAAAGAGCT	6480
CTTATACTGT GGTATTATTCT GGTGTTTAC ATTGACAGGA GACACACTGA AATCAGCAA	6540
GGAAACAGGC ATCTAAGTGG GGATGTGAAG AAAACAGGGA AAATCTTTCA GTTGTTTTCT	6600
CCCAGTGGGG TGTGTGGAC AGCACTTAAA TCACACAGAA GTGATGTGTG ACCTGTGTA	6660
TGAAGTATTT CCAACTAAGG AAGCTCACCT GAGCCTTAGT GTCCAGAGTT CTTATTGGGG	6720

GTCTGTAGGA TAGGCATGGG GTACTGGAAT AGCTGACCTT AACTTCTCAG ACCTGAGGTT	6780
CCCAAGAGTT CAAGCAGATA CAGCATGGCC TAGAGCCTCA GATGTACAAA AACAGGCATT	6840
CATCATGAAT CGCACTGTTA GCATGAATCA TCTGGCACGG CCCAAGGCC CAGGTATACC	6900
AAGGCACTTG GGCCGAATGT TCCAAGGGAT TAAATGTCAT CTCCCAGGAG TTATTCAAGG	6960
GTGAGCCCTG TACTTGGAAC GTTCAGGCTT TGAGCAGTGC AGGGCTGCTG AGTCAACCTT	7020
TTACTGTACA GGGGGGTGAG GGAAAGGGAG AAGATGAGGA AACCGCCTAG GGATCTGGTT	7080
CTGTCTTG TG GCGGAGTGGA CCATGGGGCT ATCCCAAGAA GGAGGAATTC	7130

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 858 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGAGCGGCC C TCAGCTTGC GCGGCCAGC CCCGCAAGGC TCCCGGTGAC CACTAGAGGG	60
CGGGAGGAGC TCCTGGCCAG TGGTGGAGAG TGGCAAGGAA GGACCCTAGG GTTCATCGGA	120
GGCCAGGTTT ACTCCCTTAA GTGGAAATTT CTTECCCCAC TCCTCCTTGG CTTTCTCCAA	180
GGAGGGAACC CAGGCTGCTG GAAAGTCCGG CTGGGGGGGG GACTGTGGGT TCAGGGGAGA	240
ACGGGGTGTG GAACGGGACA GGGAGCGGTT AGAAGGGTGG GGCTATTCCG GGAAGTGGTG	300
GGGGGAGGGA GCCCAAACCT AGCACCTAGT CCACTCATT TCCAGCCCTC TTATTTCTCG	360
GCGCTCTGC TTCAGTGGAC CCGGGGAGGG CGGGGAAGTG GAGTGGGAGA CCTAGGGGTG	420
GGCTTCCCGA CCTTGCTGTA CAGGACCTCG ACCTAGCTGG CTTTGTTCCT CATCCCCACG	480
TTAGTTGTTG CCCTGAGGCT AAAACTAGAG CCCAGGGGCC CCAAGTTCCA GACTGCCCCCT	540
CCCCCTCC CCGGAGCCAG GGAGTGTTG GTGAAAGGGG GAGGCCAGCT GGAGAACA	600
CGGGTAGTCA GGGGGTTGAG GATTAGAGCC CTTGTACCCT ACCCAGGAAT GGTGGGGAG	660
GAGGAGGAAG AGGTAGGAGG TAGGGGAGGG GGCGGGGTTT TGTCACCTGT CACCTGCTCG	720
CTGTGCCTAG GCGGGCGGG CGGGGAGTGG GGGGACCGGT ATAAAGCGGT AGGCGCCTGT	780
GCCCGTCCA CCTCTCAAGC AGCCAGCGCC TGCCTGAATC TGTTCTGCCC CCTCCCCACC	840
CATTTCACCA CCACCATG	858

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1581 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CATGGTGTCC GACTTATGCC CGAGAAGATG TTGAGCAAAC TTATCGCTTA TCTGCTTCTC	60
ATAGAGTCTT GCAGACAAAC TGCGCAACTC GTGAAAGGTA GCGGATCTG GGTGACCTG	120
CAGGTCAACG GATCCCTTCT TGACCAAGTAT AGCTGCATTC TTGGCTGGGG CATTCCAAC	180
AGAACTGCCA AATTAGCAC ATAAAAATAA GGAGGCCCCAG TTAAATTGGA ATTCAGATA	240
AACAATGAAT AATTGTAG TATAAATATG TCCCATGCAA TATCTTGTG AAATTAAAAA	300
AAAAAGTCTT CCTTCATGC CCCACCCCTA CCACTAGGCC TAAGGAATAG GGTGAGGGG	360
TCCAAATAGA ATGTGGTTGA GAAGTGAAT TAAGCAGGCT AATAGAAGGC AAGGGGCAA	420
GAAGAAACCT TGAATGCATT GGGTGTGGG TGCCTCCTTA AATAAGCAAG AAGGGTGCAT	480
TTTGAAGAAT TGAGATAGAA GTCTTTTGG GCTGGGTGCA GTTGCTCGTG GTTGTAATTC	540
CAGCACTTTG GGAGGCTGAG GCGGGAGGAT CACCTGAGGT TGGGAGTTCA AGACCAGCCT	600
CACCAACGTG GAGAACCCTG TCTTTACTAA AAATACAAA AATTCAGCTG GTCATGGTGG	660
CACATGCCTG TAATCCAGC TGCTCGGAG GCTGAGGCAG GAGAATCACT TGAACCAGGG	720
AGGCAGAGGT TGTGGTGAGC AGAGATCGC CCATTGCTCT CCAGCCTGGG CAACAAGAGC	780
AAAAGTTCGT TTAACAAAAA AAAAAGTCC TTTCGATGTG ACTGTCTCCT CCCAAATTTG	840
TAGACCTCT TAAGATCATG CTTTTCAGAT ACTTCAAAGA TTCCAGAAGA TATGCCCCGG	900
GGGTCCTGGA AGCCACAAGG TAAACACAAC ACATCCCCCT CCTTGACTAT CAATTTTACT	960
AGAGGATGTG GTGGGAAAC CATTATTTGA TATTAAACA AATAGGCTTG GGATGGAGTA	1020
GGATGCAAGC TCCCCAGGA AGTTAAGAT AAAACCTGAG ACTTAAAAGG GTGTTAAGAG	1080
TGGCAGCCTA GGAATTTAT CCGGACTCC GGGGAGGGG GCAGAGTCAC CAGCCTCTGC	1140
ATTTAGGGAT TCTCCGAGGA AAAGTGTGAG AACGGCTGCA GGCAACCCAG GCGTCCCGGC	1200
GCTAGGAGGG ACGACCCAGG CTGCGCGAA GAGAGGGAGA AAGTGAAGCT GGGAGTTGCC	1260
GACTCCAGGA CTTCGTTGGA ATGCAGTTGG AGGGGCGAG CTGGGAGCGC GCTTGCTCCC	1320
AATCACAGGA GAAGGAGGAG GTGGAGGAGG AGGGCTGCTT GAGGAAGTAT AAGAATGAAG	1380
TTGTGAAGCT GAGATCCCC TCCATTGGGA CCGGAGAAAC CAGGGGAGCC CCCCGGGCAG	1440

CCGCGCGCCC	CTTCCCACGG	GGCCCTTTAC	TGCGCCGCGC	GCCCGGCCCC	CACCCCTCGC	1500
AGCACCCCGC	GCCCCGCGCC	CTCCCAGCCG	GGTCCAGCCG	GAGCCATGGG	GCCGGAGCCG	1560
CAGTGAGCAC	CATGGAGCTG	G				1581

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1305 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1204..1284

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGATCCGTCC	CGGGACTAGC	AGGGCTTTGG	GCAGCAACCC	GCAGGAGCCC	GACCGCCTCT	60
GGCCAGGTCC	GGGCAGCTGG	TGGGGGAGGT	TCCAGAGGTC	CACGCCATTG	GTGGACGCAG	120
TCTCTAGTGT	CCTCTCCGCG	TCCCACTTCA	CTGCCCCATC	CCCTTTCCTG	CGAGAGCCTG	180
GACTTGAAG	GCACCTGGGA	GGGTGTAAGC	GCCTTGGTGT	GTGCCCCTCT	GGGTCCCCAG	240
AAGAGCGGCG	GGAAGTCCGG	CGGCCCGGAC	GGTGCAGCCA	GAATCCAGTG	TGGAAGGGGA	300
GGCAGCTGTT	CTCCCAGGCG	GCCGTGGGGG	GCAGCAGAGG	GGACGGCGAC	AGGTGCGGGA	360
GCCCCCTCCC	GGGTAGAAGT	GGAAAGGCGG	GCTCCGGGGT	CTGTTCCAG	GCTGGAAACC	420
ACCCCCGCCC	CCCATCCAAA	TCCCCGGGAG	AGGCCCCGCC	GGCGCCGGGT	CTGGAGGAGG	480
AAGCGGCCAG	AGACAGTGCA	ATTTACGCG	GTCTCTGTGG	CTCGGGTTCC	TGGGCTGGGT	540
GGATGAATTA	TGGGGTTTCG	AGTCTGGGAG	AAACTGAGGT	GGCCTGGACG	TGAGGCAAAA	600
AACACCCCTC	CCCTCAAAAA	CACACAGAGA	GAAATATTCA	CATTCTGAGA	GAAAATCCAC	660
CAAGTGAACC	AACCGGCTAG	GGGAGTTGAG	TGATTTGGTT	AATGGGCGAG	GCCAACTTTC	720
AGGGGGCAGG	GCTTTGGAGA	GCTTTCCACT	CCCTCATTCA	TTACCCTTCC	CTGGATCTGG	780
GGGCTTTTCG	AATCTCGACC	TCCCCTTGGC	CTATCTCCTG	CAGAAAAATT	AGGGTGAGCC	840
CCATCCTCGA	TCTGCTCCGC	CAAGTTGCGG	GACCGCGGGG	CGTGGCACGC	TCAGGGGCAG	900
GCGGTCCGAG	GCTCCGCAAT	CCCCACTCCA	GCCTCGCGCG	GGAGGGGGCG	CGGCCCGTGT	960
GACTCACCCC	CTTCCCTCTG	CGTTCCTCCC	TCCCTCTCTC	TCTCTCTCTC	ACACACACAC	1020
ACCCCTCCCC	TGCCATCCCT	CCCCGGACTC	CGGCTCCGGC	TCCGATTGCA	ATTGCAACC	1080

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TCCGCTGCCG TCGCCG CAGC AGCCACCAAT TCGCCAGCGG TTCAGGTGGC TCTTGCCTCG 1140
 ATGTCCTAGC CTAGGGGCCC CCGGGCCGGA CTTGGCTGGG CTCCCTTCAC CCTCTGCGGA 1200
 GTC ATG AGG GCG AAC GAC GCT CTG CAG GTG CTG GGC TTG CTT TTC AGC 1248
 Met Arg Ala Asn Asp Ala Leu Gln Val Leu Gly Leu Leu Phe Ser
 1 5 10 15
 CTG GCC CGG GGC TCC GAG GTG GGC AAC TCT CAG GCA GGTAAGTGCC 1294
 Leu Ala Arg Gly Ser Glu Val Gly Asn Ser Gln Ala
 20 25
 CAGAGAGCAC C 1305

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Arg Ala Asn Asp Ala Leu Gln Val Leu Gly Leu Leu Phe Ser Leu
 1 5 10 15
 Ala Arg Gly Ser Glu Val Gly Asn Ser Gln Ala
 20 25

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4752 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATTCATGCC CCAGTTGACA ACATAGCTGG TTCAAGACAA ACCTGAGCAC TTTTCATCAC 60
 TGAAATCTTC ACTCTGGACC AATCAACATT CATAATTCC CTTCTTTACT TTAACACTCT 120
 CCTGAGAGCT ATTTCTCTTC TCATCCTAAT TCTCTGCTCA TATCACATT CAGCAGCTTA 180
 CATATGAAAA TCTTGACAT TCCCATGAGA TTGCATTGAA ATTGCTTCAA CCGTTTTCTA 240
 TGTCCATATG TATACCTTAC TTCTATTCC TATTGCTTTG TAGCTACAGA AGCCTTAGCA 300
 GTCTTTAGAA ATCTTGGGAG TGTTATGCCT CTTCCACTTA AAGCTTTAAT TCAAGAACTC 360
 ATTCTCTAGA AGTTTAAACA ACTGCTATTG TGCTCTTCTA TGAATCTTTA ACATGTCTCT 420
 CAAATATGT TTTCTCCCAG AAAACTCTCC TCAACTTTCT TCAAAATTAA TAGACTACAT 480

TTTGCCCCAC AGAAATTCTT CTATAGGAAC TTCAATTTTT TTATTTAATC CAGGAAAACA	540
TATTTAAACA TACTTAGCTA TAAATATTAA GATTAAATTG TGATAAAAAC TTATTTTGA	600
CAGTGGTGGG AACTGTCCTA TTAAATTTAC CATATCATTG CAATTTTATC CTCAGAAAAT	660
CCCCAATAGA TGATCAATAA ATATTTGCAG TATACTTATT GCATTCCCAG CTATAGCTAA	720
TATAGATTGC ATTAAGACTG TCTTAGGGTA GACAATAGTC AAACAGAATT GGATAGGGCA	780
TATTCAAATT CTGTAGCTAC CAAATGTGTA TACAAGGGAA AAATGTGTAA ATGAATACTT	840
ACATAGAAAG AGCAGGACTG GTGATAAGAT TGTCATGTAA ATAAGTTGGT GGAGACTTGA	900
GTTACATTGC AGAGGAATAA AGAGAGCTTT TAATTTAAGA GAATCAGTCC TATGAAGAAA	960
ACAAAGACAG CAAACTACAC TTCAGTCATT CTCTCATGAG GCTCTACAGA TTTTGAAAAT	1020
GAGCATGGGA AAGCCTTAGA AAATGAGCAT GGGAAAGCCC TTAGAAAGAG TGATGAGGAT	1080
TTTCTGAAAT TTTGCTAAGA TGCCTGATAG TAGAACAAGG GAAAGAAAAA GTTGTTAATT	1140
AAGTTTAACA GGAGAAGCTG GATTCCACCT GCACTAGGT GGGAAAGAGT TACAAAAGGC	1200
CCCATGTAGC TACAAGTAAT ACATAACAAA AAATCCCCAG GGGAAACGAG GTCCTGTGTT	1260
AATCTACTAT GGGCTTTAAG AGAAGAAAAA CAAAAAGGA CTAAGACTCT GAAGGAAATC	1320
ATGTGAGAGC TTCCTTATTC CAGCAAGAGA CAAAATCTCC ATGGAAACTT CTCTTTCCTG	1380
CACCCACACA CTGTTCTTTC TACCTCGCAA GGCTGCCTTG AATCTCAAGA GAATCCTATG	1440
GAGCAGTCAA CACATTTTAA ATACTGAAAC AAACCCTAGG GAAGAAAAGA AGCAGAGCAG	1500
GCTGACATTC CAGCATTATC AGGAAAGCAA TGATTTTCCT AGATTTCCGC AGCCCCAGTG	1560
TTCAGATAAA CGGTTTCCTC AAACCTTCAC TTCCTTTCCTC CTACAGTATA AATTAAAAGA	1620
ACCACTCAGC TTTTAGTATG AAGCAGCATA GAGAAGGGAG TTCCAAAGAG ACGTTTGTTT	1680
CTTGACCATT CTCATTATCC TTCTTTCATG GAGCAGTGCT ATTCAAACCA TCCAGTAAGT	1740
CCATTACTCA CTTCCACATT TTATGAGCAA AATAATAAAA GAGAGATAGA GTAAGAATGA	1800
AGGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGGAAGA	1860
GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGG	1920
TGTTTTCTTA ACTAGAACT TTATGCATTG AAGCAGTTCA CAAAATAAC AAAGTAACAA	1980
AGTAAGATAT CTTTGGAATA ATCAATTCAA GATAATCAAG GAAAAATGAG AGGCAACTAT	2040
TTTAGACTGA TTACTTTTAT AAAATAAATA AGCTCAGCTT AGCCAGATAT AAGCAATATT	2100
CTGAGTTCTG AAGAAAAATT TTTGACAAA TGAGTTCTAT AAATGTTATT GTCTACTTAT	2160
GATCTCTAAA TACAACAGGC TTGTATTCAG AATCTAGATG TTTCATGACC TTTATTCATA	2220
AGAGATGATG TATTCTTGAT ACTACTTCTC ATTTGCAAAT TCCAATTATT ATTAATTCA	2280
TATCAATTAG AATAATATAT CTTCCCTTCAA TTTAGTTACC TCACTATGGG CTATGTACAA	2340
ACTCCAAGAA AAAGTTAGTC ATGTGCTTTG CAGAAGATAA AAGCTTAGTG TAAAACAGGC	2400
TGAGAGTATT TGATGTAAGA AGGGGAGTGG TTATATAGGT CTTAGCCAAA ACATGTGATA	2460

GTCACCTCCAG GGGTTGCTGG AAAAGAAGTC TGTGACACTC ATTAACCTAT TGGTGCAGAT	2520
TTTGTATGAT CTAAAGGAGA AAATGTTCTT GGCTGTTTTG TATTGCCTTC TGTGGAGTTT	2580
CCAGATCTCT GATGGCCATT TTCCTCGAGC CTGTGCCTCC TCTAAGAACT TGTTGGCAAA	2640
AGAATGCTGC CCACCATGGA TGGGTGATGG GAGTCCCTGC GGCCAGCTTT CAGGCAGAGG	2700
TTCCTGCCAG GATATCCTTC TGTCCAGTGC ACCATCTGGA CCTCAGTTCC CCTTCAAAGG	2760
GGTGGATGAC CGTGAGTCCT GGCCCTCTGT GTTTTATAAT AGGACCTGCC AGTGCTCAGG	2820
CAACTTCATG GGTTCACACT GCGGAAACTG TAAGTTTGGA TTTGGGGGCC CAAATTGTAC	2880
AGAGAAGCGA GTCTTGATTA GAAGAAACAT TTTGATTTG AGTGTCTCCG AAAAGAATAA	2940
GTCTTTTCT TACCTCACTT TAGCAAAACA TACTATCAGC TCAGTCTATG TCATCCCCAC	3000
AGGCACCTAT GGCCAAATGA ACAATGGGTC AACACCCATG TTTAATGATA TCAACATCTA	3060
CGACCTCTTT GTATGGATGC ATTACTATGT GTCAAGGGAC AACTGCTTG GGGGCTCTGA	3120
AATATGGAGG GACATTGATT TTGCCCATGA AGCACCAGGG TTTCTGCCTT GGCACAGACT	3180
TTTCTTGTTA TTGTGGGAAC AAGAAATTCG AGAACTAACT GGGGATGAGA ACTTCACTGT	3240
TCCATACTGG GATTGGAGAG ATGCAGAAAA CTGTGACATT TGCACAGATG AGTACTTGGG	3300
AGGTGCTCAC CCTGAAAATC CTAACCTACT CAGCCCAGCA TCCTTCTTCT CCTCCTGGCA	3360
GGTAAGATGC ACTATATAGA GAGAGTTGCA AAGACTGGTA CTTCAGCAGC CACATTTTCA	3420
TGCTCTGTGA GCATCTCTGA TAATATCTCA GGGCAGAAAA TGTGCCTTAC TAACAGATGT	3480
TAATGCTTCT TGATTTCTTT TTCTCTTTG AGAACTCTTC AAAGTGTTAT TAAACAAATA	3540
TCTATGTGCT TATTTGTCTT AATATCTAAC AGCTTAGTTA GATTTCTAAG CTGCTATAAA	3600
CAAGGACTGA TTGGTTCACC ACTGTATTGT TAGCACCTCC TATGTATCTA ATAACAGTAA	3660
CTCAGTTATT AAGATGGAT AGAAACCAGA TTATCTTAGT TCAATTTCTA GTAATATTAA	3720
ACTTAATATA ACAGTAAATC CATAAGTATC TACTTAAAT ATAATCTCTG GCCAAACCAA	3780
GACTTATTAT TAGGATCTTC AAGAGAAAGT GCTGAGATAA TTCACTAAGT ATCAGAGATG	3840
ACCTTTATTA CATGATTGCC TGATAGAAAA AATGATTACA CACACACAAA AAAATCTTCA	3900
GTTGCTTAAT TTAAGCGCTG ACTCTCAACA GTTAAGTAAT AAAAGAGTTA AGCCTGCTGT	3960
GTATTTAGAA TATGTGAATA CCTATTGAAA GAATTTATTG TACAATTAAT ATAAACAGAC	4020
TTCTATTTTA CATCATAAGA TACTACTTAA TTTGTTAAAA ATTATTTTTT ATACATTGTT	4080
GTAAATACAA AGTGATATTT CTAATGATTA CAAGGCTGTC TGGCTAACTT ACGTTATGTT	4140
CAGGAGAAGA CAGTCCTTTT TAAGGAATGG GCACCTTCTA ACTTTTTTTC TCTAGGATGG	4200
AGAAAAATTA GCCTTCTTCC TACTTTAAAA ATGTTAGACA TAGAATTAAG GGATTGTTAT	4260
TTTGAGATTA AATTTTCTTT TCTCCTATTA TTTTTCCTCA TTCTGGAATG GAAGCAAAAG	4320
ATGAAGAAAG AAATATATGT TAAATTGTTT TCCTTTAAAT GAACACAAAT GTGAAATATG	4380
TTTTTCTGCC TATCTTGTA AATTTTCTAT TGCAACTATT CTGATTACCA GTTCAAATGG	4440

GGAAAAAAGA ACATAGGCTA CCCACACTT GAAATTTTGA AATATGAATG TCCTCTGTCT	4500
CTAGCTGAGT ACTCTGGCGC TTCCAAAATG GAAACCTTTA AAGGGCCACT GTAAATTACA	4560
GCTGCTAATT CCTGGTGCCA ATGGTGATAA GTGTTTACTA AACCTAGTGA GTACTTTATA	4620
GCATGGGTCT GCTGCGAAGT AACATTGCTG TATATTTTCA GTCATTCTAC CTTAATTCAT	4680
GAACGCAAA ACTCTCATCT AGCTTTTAC TTCTCTAGCT ATTGCTTTAA GTTCTATCAG	4740
GCTCAGGTGT GG	4752

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1236 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAGCTTTGTA GAGTAATCAT GTATTCCAAA CTCAGGCTTA CATTGAATG TTGGCTACAT	60
ATGTATGAGT TTTCAACTTC CAGGAGAAAA CGTCTCTTTA AAAGAGAACA ACCAAAAGCT	120
AACAGAAAAT ACAAGTGTGA CATTGGCCTT AGTTCGACCA AGAAAGCAAT TCATCTTGTT	180
TCTTCCTTG TGGTATACAG ATAAGAAAA TAAATCACT ACAACGAAG CAAAATCTCT	240
TCAGCGTCTC TAATACATCT TCCAAATCAG TGTGTCTGAC CTTTTCTTAA GACTTTAACC	300
ATCACAAGGA AACCAGTGGG GAGGGAGTCA TGTGCTGCCT AGTAGTTAA GGGCAGGAGA	360
ATCACTGGT GTGAGAAGGG ATTAGTGAGA GCTGGAAGAG AGGACCAGCC CCTCCCAGTG	420
TGAGGAATCT GGCTTGGGAT TTAAGTCTG GCAGAAAATC TCTTCGGGCA ATTAACAGCT	480
GGCATCAGGG GAAAAGCAGA CATCCAACAA CACTAGCTCT GAAGGAGATC AGCAGAGAAA	540
CCTCCACGG ATTCAATGTA CTGGTGAGCA GCTCTGTGGT GGGTACCCTT GTGACCAAAG	600
CTCTAGGAAC ATGAAGGAGA TTTGCTTGCT ATAAACCTGT TTCCTATTCT CTTTCATT	660
CCATGGTTAA CTATTACTAT GGTAGTCACC AACTAGTGGA TGCTTTTGGT AAATGACATC	720
TATGGAAAGT CTTTTTGGAT CAGGGTGATC TTTTATGTA TGTGTATGTG CATGGATATG	780
GGTGACAGAG AGCAGGTGCC CAGATTCTCA AGGAGGGCTT CAGTTACAAG GAGTTGGGAG	840
TGATCTGATG TGGTTGCAAG GCACTGAAGT CAGTCTCTCT GTAAGAGCAC TCTATGCTCC	900
TTACCACTGT GCCTTCTCCC CAGCCCAAGA ATAGTATTCT TATGGGTAGA AATTTAAATA	960
AGAAACTCAA AGACCAGGAG AGTGAGTTCT GTCATCTAGC TATTATGCCT GCAGATATT	1020
AAAGGTGAAT AATTATTTTG ACTATTGTTT AGAAATGTTG TTTACATGA AAGATTCCAT	1080

TTCCGGAGTG GGTGAAAAG TATGCAAAG AACTTTTGCA ACTCTGTTT TGCCTTTCTG	1140
TTTTTCAGCT GTATTTTCAT CTGAGCACCC CTGTCTTCTC CATGCAAAGA GCAGCATAGG	1200
AGACCTGTGT TCTGAACTCT TGCTTCGAGA AGAATG	1236

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5737 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTAATCAACA AATCTAAACA TTTATTCTTT TCATCTGTTT ACTCTTGCTC TTGTTACCA	60
CAATATGCTA TTCACATGTT CAGTGTAGTT TTATGACAAA GAAAATTTTC TGAGTTACTT	120
TTGTATCCCC ACCCCCTTAA AGAAAGGAGG AAAAAGCTT TCATACAGAA GGCCTTAATT	180
GCATGAATTA GAGCTATCAC CTAAGTGTGG GCTAATGTAA CAAAGAGGGA TTTCACCTAC	240
ATCCATTCAG TCAGTCTTTG GGGGTTTAAA GAATTCCTAA GAGTCATCAG AAGAGGAAAA	300
ATGAAGGTAA TGTTTTTTCA GACAGGTAAA GTCTTTGAAA ATATGTGTAA TATGTAAAAC	360
ATTTTGACAC CCCATAATA TTTTCCAGA ATTAACAGTA TAAATTGCAT CTCTTGTTCA	420
AGAGTTCCTT ATCACTCTCT TTAATCACTA CTCACAGTAA CCTCAACTCC TGCCACAATG	480
TACAGGATGC AACTCCTGTC TTGCATTGCA CTAAGTCTTG CACTTGTCAC AAACAGTGCA	540
CCTACTTCAA GTTCTACAAA GAAACACAG CTACAAGTGG AGCATTACT GCTGGATTA	600
CAGATGATTT TGAATGGAAT TAATGTAAGT ATATTTCTT TCTTACTAAA ATTATTACAT	660
TTAGTAATCT AGCTGGAGAT CATTCTTAA TAACAATGCA TTATACTTTC TTAGAATTAC	720
AAGAATCCCA AACTCACCAG GATGCTCACA TTTAAGTTT ACATGCCCAA GAAGGTAAGT	780
ACAAATATTT ATGTTCAATT TCTGTTTTAA TAAAATTCAA AGTAATATGA AAATTGTCAC	840
AGATGGGACT AATAGCAGCT CATCTGAGGT AAAGAGTAAC TTTAATTGT TTTTTTGAAA	900
ACCCAAGTTT GATAATGAAG CCTCTATTAA AACAGTTTTA CCTATATTTT TAATATATAT	960
TTGTGTGTTG GTGGGGGTGG GAAGAAAACA TAAAAATAAT ATTCTCACCT TTATCGATAA	1020
GACAATTCTA AACAAAAATG TTCATTATG GTTTCATTTA AAAATGTAA ACTCTAAAAT	1080
ATTTGATTAT GTCATTTTAG TATGTAAAAT ACCAAAATCT ATTTCCAAGG AGCCCACTTT	1140
TAAAAATCTT TTCTTGTTTT AGGAAAGGTT TCTAAGTGAG AGGCAGCATA AACTAATAG	1200
CACAGAGTCT GGGGCCAGAT ATCTGAAGTG AAATCTCAGC TCTGCCATGT CCTAGCTTTC	1260

ATGATCTTTG GCAAATTACC TACTCTGTTT GTGATTCACT TTCATGTCTA CTTAAATGAA	1320
TAACTGTATA TACTTAATAT GGCTTTGTGA GAATTAGTAA GTTAAATGTA AAGCACTCAG	1380
AACCGTGTCT GGCATAAGGT AAATACCATA CAAGCATTAG CTATTATTAG TAGTATTAAA	1440
GATAAAATTT TCACTGAGAA ATACAAAGTA AAATTTTGGG CTTTATCTTT TTACCAATAG	1500
AACTTGAGAT TTATAATGCT ATATGACTTA TTTTCCAAGA TTAAAAGCTT CATTAGGTTG	1560
TTTTTGGATT CAGATAGAGC ATAAGCATAA TCATCCAAGC TCCTAGGCTA CATTAGGTGT	1620
GTAAAGCTAC CTAGTAGTTG TGCCAGTTAA GAGAGAATGA ACAAATCTG GTGCCAGAAA	1680
GAGCTTGTGC CAGGGTGAAT CCAAGCCCAG AAAATAATAG GATTTAAGGG GACACAGATG	1740
CAATCCCAT T GACTCAAATT CTATTAATTC AAGAGAAATC TGCTTCTAAC TACCCTTCTG	1800
AAAGATGTAA AGGAGACAGC TTACAGATGT TACTCTAGTT TAATCAGAGC CACATAATGC	1860
AACTCCAGCA ACATAAGAT ACTAGATGCT GTTTTCTGAA GAAAATTTCT CCACATTGTT	1920
CATGCCAAAA ACTTAAACCC GAATTTGTAG AATTTGTAGT GGTGAATTGA AAGCGCAATA	1980
GATGGACATA TCAGGGGATT GGTATTGTCT TGACCTACCT TTCCCCTAA AGAGTGTTAG	2040
AAAGATGAGA TTATGTGCAT AATTTAGGGG GTGGTAGAAT TCATGGAAAT CTAAGTTTGA	2100
AACCAAAAGT AATGATAAAC TCTATTCATT TGTTCATTTA ACCCTCATTG CACATTTACA	2160
AAAGATTTTA GAAACTAATA AAAATATTTG ATTCCAAGGA TGCTATGTTA ATGCTATAAT	2220
GAGAAAGAAA TGAAATCTAA TTCTGGCTCT ACCTACTTAT GTGGTCAAAT TCTGAGATTT	2280
AGTGTGCTTA TTTATAAAGT GGAGATGATA CTTCACTGCC TACTTCAAAA GATGACTGTG	2340
AGAAGTAAAT GGGCCTATTT TGGAGAAAAT TCTTTTAAAT TGTAATATAC CATAGAAATA	2400
TGAAATATTA TATATAATAT AGAATCAAGA GGCCTGTCCA AAAGTCCTCC CAAAGTATTA	2460
TAATCTTTTA TTCACTGGG ACAACATTT TTAATGCA TCTTAATGTA GTGATTGTAG	2520
AAAAGTAAAA TTAAAGACAT ATTTAAAAAT GTGTCTTGCT CAAGGCTATA TTGAGAGCCA	2580
CTACTACATG ATTATTGTTA CCTAGTGTA AATGTTGGGA TTGTGATAGA TGGCATTCAA	2640
GAGTTCCTTC TCTCTCAACA TTCTGTGATT CTTAACTCTT AGACTATCAA ATATTATAAT	2700
CATAGAATGT GATTTTATG CTTCCACATT CTAATCATC TGGTTCTAAT GATTTTCTAT	2760
GCAGATTGGA AAAGTAATCA GCCTGCATCT GTGATAGGCA CTTACGATGC AGAAAGTCTA	2820
ACATTTTGCA AAGCCAAATT AAGCTAAAC CAGTGAGTCA ACTATCACTT AACGCTAGTC	2880
ATAGGTACTT GAGCCCTAGT TTTTCCAGTT TTATAATGTA AACTCTACTG GTCCATTCTT	2940
TACAGTGACA TTGAGAACAG AGAGAATGGT AAAAATACA TACTGCTACT CCAAATAAAA	3000
TAAATTGGAA ATTAATTTCT GATTCTGACC TCTATGTAAA CTGAGCTGAT GATAATTATT	3060
ATTCTAGGCC ACAGAACTGA AACATCTTCA GTGTCTAGAA GAAGAACTCA AACCTCTGGA	3120
GGAAGTGCTA AATTTAGCTC AAAGCAAAA CTTTCACTTA AGACCCAGGG ACTTAATCAG	3180
CAATATCAAC GTAATAGTTC TGGAATAAAA GGTAAGGCAT TACTTTATTT GCTCTCCTGG	3240

AAATAAAAAA AAAAAAGTCA GGGGGAAAAG TACCACATTT TAAAGTGACA TAACATTTTT	3300
GGTATTTGTA AAGTACCCAT GCATGTAATT AGCCTACATT TTAAGTACAC TGTGAACATG	3360
AATCATTTCT AATGTTAAAT GATTAAGTGG GGAGTATAAG CTACTGAGTT TGCACCTACC	3420
ATCTACTAAT GGACAAGCCT CATCCCAAAC TCCATCACCT TTCATATTAA CACAAAACCTG	3480
GGAGTGAGAG AAGGTACTGA GTTGAGTTTC ACAGAAAGCA GGCAGATTTT ATTATATATT	3540
TTTCGATTCT TCAGATCATT TACTGAAATA GCCAATACTG ATTACCTGAA AGGCTTTTCA	3600
AATGGTGTTT CCTTATCATT TGATGGAAGG ACTACCCATA AGAGATTTGT CTTAAAAAAA	3660
AAAACCTGGAG CCATTAAAT GGCCAGTGGG CTAAACAAAC AACAACTTTT TTAGAGGCAA	3720
TCCCCACTTT CAGAATCTTA AGTATTTTTTA AATGCACAGG AAGCATAAAA TATGCAAGGG	3780
ACTCAGGTGA TGTAAGAGAG ATTCACTTTT GTCTTTTTAT ATCCCGTCTC CTAAGGTATA	3840
AAATTCATGA GTTAATAGGT ATCCTAAATA AGCAGCATAA GTATAGTAGT AAAAGACATT	3900
CCTAAAAGTA ACTCCAGTTG TGTCCAAATG AATCACTTAT TAGTGGACTG TTCAGTTGA	3960
ATTAAAAAAA TACATTGAGA TCAATGTCAT CTAGACATTG ACAGATTCAG TTCCTTATCT	4020
ATGGCAAGAG TTTTACTCTA AAATAATTAA CATCAGAAAA CTCATTCTTA ACTCTTGATA	4080
CAAATTTAAG ACAAACCAT GCAAAAATCT GAAAACGTG TTTCAAAGC CAAACACTTT	4140
TTAAATATAA AAATCCCAAG ATATGACAAT ATTTAAACAA TTATGCTTAA GAGGATACAG	4200
AACACTGCAA CAGTTTTTTA AAAGAGAATA CTTATTTTAA GGGAACTCTC TATCTCACCT	4260
GCTTTTGTTT CCAGGGTAGG AATCACTTCA AATTTGAAAA GCTCTCTTTT AAATCTCACT	4320
ATATATCAAA ATATTTCTCT CTTAGCTTAT CAACTAGAGG AAGCGTTTAA ATAGCTCCTT	4380
TCAGCAGAGA AGCCTAATTT CTAAAAAGCC AGTCCACAGA ACAAATTTT TAATGTTTAA	4440
ACTTTTAAAA GTTGGCAAAT TCACCTGCAT TGATACTATG ATGGGGTAGG GATAGGTGTA	4500
AGTATTTAGA AGATGTTCTT CACACAAATT TATCCCAAAC GGAAGCATGT CCTAGCTTAC	4560
TCTAGTGTAG TTCTGTTCTG CTTGGGGGAA AATATAAGGA GATTCACTTA AGTAGAAAAA	4620
TAGGAGACTC TAATCAAGAT TTAGAAAAGA AGAAAGTATA ATGTGCATAT CAATTCATAC	4680
ATTTAACTTA CACAAATATA GGTGTACATT CAGAGGAAAA GCGATCAAGT TTATTTTACA	4740
TCCAGCATTT AATATTTGTC TAGATCTATT TTTATTTAAA TCTTTATTTG CACCCATTT	4800
AGGGAAAAAA TTTTGTGTT CATTGACTGA ATTAACAAAT GAGGAAAATC TCAGCTTCTG	4860
TGTTACTATC ATTTGGTATC ATAACAAAT ATGTAATTTT GGCATTCATT TTGATCATTT	4920
CAAGAAAATG CGAATAATTA ATATGTTTGG TAAGCTTGAA AATAAAGGCA ACAGGCCTAT	4980
AAGACTTCAA TTGGGAATAA CTGTATATAA GGTAACTAC TCTGTACTTT AAAAAATTAA	5040
CATTTTTCTT TTATAGGGAT CTGAAACAAC ATTCATGTGT GAATATGCTG ATGAGACAGC	5100
AACCATTGTA GAATTTCTGA ACAGATGGAT TACCTTTTGT CAAAGCATCA TCTCAACACT	5160
GACTTGATAA TTAAGTGCTT CCCACTTAAA ACATATCAGG CCTTCTATTT ATTTAAATAT	5220

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TTAAATTTTA TATTTATTGT TGAATGTATG GTTGCTACC TATTGTAAC ATTATTCTTA	5280
ATCTTAAAAC TATAAATATG GATCTTTTAT GATTCTTTT GTGCCCTAGG GGCTCTAAAA	5340
TGGTTTCACT TATTTATCCC AAAATATTTA TTATTATGTT GAATGTTAAA TATAGTGCTA	5400
TGTAGATTGG TTAGTAAAAC TATTTAATAA ATTTGATAAA TATAACAAG CCTGGATATT	5460
TGTTATTTTG GAAACAGCAC AGAGTAAGCA TTAAATATT TCTTAGTTAC TTGTGTGAAC	5520
TGTAGGATGG TTAAAATGCT TACAAAAGTC ACTCTTCTC TGAAGAAATA TGTAGAACAG	5580
AGATGTAGAC TTCTCAAAAG CCCTTGCTTT GTCCTTTCAA GGGCTGATCA GACCCTTAGT	5640
TCTGGCATCT CTTAGCAGAT TATATTTTCC TTCTTCTTAA AATGCCAAAC ACAAACACTC	5700
TTGAAACTCT TCATAGATTT GGTGTGGCTA TGAATTC	5737

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 614 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GATCGTTAGC TTCTCCTGAT AACTAATTG CCTCACATTG TCACTGCAA TCGACACCTA	60
TTAATGGGTC TCACCTCCCA ACTGCTTCCC CCTCTGTTCT TCCTGCTAGC ATGTGCGGGC	120
AACTTTGTCC ACGGACACAA GTGCGATATC ACCTTACAGG AGATCATCAA AACTTTGAAC	180
AGCCTCACAG AGCAGAAGAC TCTGTGCACC GAGTTGACCG TAACAGACAT CTTTGCTGCC	240
TCCAAGAACA CAACTGAGAA GGAACCTTC TGCAGGGCTG CGACTGTGCT CCGGCAGTTC	300
TACAGCCACC ATGAGAAGGA CACTCGCTGC CTGGGTGCGA CTGCACAGCA GTTCCACAGG	360
CACAAGCAGC TGATCCGATT CCTGAAACGG CTCGACAGGA ACCTCTGGGG CCTGGCGGGC	420
TTGAATTCCT GTCCTGTGAA GGAAGCCAAC CAGAGTACGT TGGAAAAC TTGGAAGG	480
CTAAAGACGA TCATGAGAGA GAAATATTCA AAGTGTTTCA GCTGAATATT TTAATTTATG	540
AGTTTTTGAT AGCTTTATTT TTAAGTATT TATATATTTA TAACTCATCA TAAATAAAG	600
TATATATAGA ATCT	614

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1589 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GAATTCCTCT GGTCTCATC CAGGTGCGCG GGAAGCAGGT GCCCAGGAGA GAGGGGATAA	60
TGAAGATTCC ATGCTGATGA TCCCAAAGAT TGAACCTGCA GACCAAGCGC AAAGTAGAAA	120
CTGAAAGTAC ACTGCTGGCG GATCCTACGG AAGTTATGGA AAAGGCAAAG CGCAGAGCCA	180
CGCCGTAGTG TGTGCCGCCC CCCTTGGGAT GGATGAAACT GCAGTCGCGG CGTGGGTAAG	240
AGGAACCAGC TGCAGAGATC ACCCTGCCCA ACACAGACTC GGCAACTCCG CGGAAGACCA	300
GGGTCTGGG AGTGACTATG GGCGGTGAGA GCTTGCTCCT GCTCCAGTTG CGGTCATCAT	360
GACTACGCCC GCCTCCCGCA GACCATGTTT CATGTTTCTT TTAGGTATAT CTTTGGACTT	420
CCTCCCCTGA TCCTTGTTCT GTTGCCAGTA GCATCATCTG ATTGTGATAT TGAAGGTAAG	480
GATGGCAAAC AATATGAGAG TGTCTAATG GTCAGCATCG ATCAATTATT GGACAGCATG	540
AAAGAAATTG GTAGCAATTG CCTGAATAAT GAATTTAACT TTTTAAAAG ACATATCTGT	600
GATGCTAATA AGGAAGGTAT GTTTTTATTC CGTGCTGCTC GCAAGTTGAG GCAATTTCTT	660
AAAATGAATA GCACTGGTGA TTTTGATCTC CACTTATTAA AAGTTTCAGA AGGCACAACA	720
ATACTGTTGA ACTGCACTGG CCAGGTAAA GGAAGAAAAC CAGCTGCCCT GGGTGAAGCC	780
CAACCAACAA AGAGTTTGGA AGAAAATAAA TCTTTAAAGG AACAGAAAAA ACTGAATGAC	840
TTGTGTTTCC TAAAGAGACT ATTACAAGAG ATAAAACTT GTTGAATAA AATTTTGATG	900
GGCACTAAG AACACTGAAA AATATGGAGT GGCAATATAG AAACACGAAC TTTAGCTGCA	960
TCCTCCAAGA ATCTATCTGC TTATGCAGTT TTTCAGAGTG GAATGCTTCC TAGAAGTTAC	1020
TGAATGCACC ATGGTCAAAA CGGATTAGGG CATTGAGAA ATGCATATTG TATTACTAGA	1080
AGATGAATAC AAACAATGGA AACTGAATGC TCCAGTCAAC AAACATTTTC TTATATATGT	1140
GAACATTTAT CAATCAGTAT AATTCTGTAC TGATTTTGT AAGACAATCC ATGTAAGGTA	1200
TCAGTTGCAA TAATACTTCT CAAACCTGTT TAAATATTTC AAGACATTAA ATCTATGAAG	1260
TATATAATGG TTTCAAAGAT TCAAAATTGA CATGCTTTA CTGTCAAAT AATTTTATGG	1320
CTCACTATGA ATCTATTATA CTGTATTAAG AGTGAAAATT GTCTTCTTCT GTGCTGGAGA	1380
TGTTTTAGAG TTAACAATGA TATATGGATA ATGCCGGTGA GAATAAGAGA GTCATAAACC	1440
TTAAGTAAGC AACAGCATAA CAAGGTCCAA GATACCTAAA AGAGATTTCA AGAGATTAA	1500
TTAATCATGA ATGTGTAACA CAGTGCCTTC AATAAATGGT ATAGCAAATG TTTGACATG	1560
AAAAAAGGAC AATTTCAAAA AATAAAAAT	1589

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1585 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CACACCCTGA CAAGCTGCCA GGCAGGTTCT CTCCTCTCA CATACTGACC CACGGCTCCA	60
CCCTCTCTCC CCTGGAAAGG ACACCATGAG CACTGAAAGC ATGATCCGGG ACGTGGAGCT	120
GGCCGAGGAG GCGCTCCCCA AGAAGACAGG GGGGCCCCAG GGCTCCAGGC GGTGCTTGTT	180
CCTCAGCCTC TTCTCCTTCC TGATCGTGGC AGGCGCCACC ACGCTCTTCT GCCTGCTGCA	240
CTTTGGAGTG ATCGGCCCCC AGAGGGAAGA GTCCCCCAGG GACCTCTCTC TAATCAGCCC	300
TCTGGCCCAG GCAGTCAGAT CATCTTCTCG AACCCCGAGT GACAAGCCTG TAGCCCATGT	360
TGTAGCAAAC CCTCAAGCTG AGGGGACAGT CCAGTGGCTG AACCGCCGGG CCAATGCCCT	420
CCTGGCCAAT GCGGTGGAGC TGAGAGATAA CCAGCTGGTG GTGCCATCAG AGGGCCTGTA	480
CCTCATCTAC TCCCAGGTCC TCTTCAAGGG CCAAGGCTGC CCCTCCACCC ATGTGCTCCT	540
CACCCACACC ATCAGCCGCA TCGCCGTCTC CTACCAGACC AAGGTCAACC TCCTCTCTGC	600
CATCAAGAGC CCCTGCCAGA GGGAGACCCC AGAGGGGGCT GAGGCCAAGC CCTGGTATGA	660
GCCCATCTAT CTGGGAGGGG TCTTCCAGCT GGAGAAGGGT GACCGACTCA GCGCTGAGAT	720
CAATCGGCCC GACTATCTCG ACTTTGCCGA GTCTGGGCAG GTCTACTTTG GGATCATTGC	780
CCTGTGAGGA GGACGAACAT CCAACCTTCC CAAACGCCTC CCCTGCCCCA ATCCCTTTAT	840
TACCCCTTCC TTCAGACACC CTCAACCTCT TCTGGCTCAA AAAGAGAATT GGGGGCTTAG	900
GGTCGGAACC CAAGCTTAGA ACTTTAAGCA ACAAGACCAC CACTTCGAAA CCTGGGATTC	960
AGGAATGTGT GGCCTGCACA GTGAAGTGCT GGCAACCACT AAGAATTCAA ACTGGGGCCT	1020
CCAGAACTCA CTGGGGCCTA CAGCTTTGAT CCCTGACATC TGGAACTCTG AGACCAGGGA	1080
GCCTTTGGTT CTGGCCAGAA TGCTGCAGGA CTTGAGAAGA CCTCACCTAG AAATTGACAC	1140
AAGTGGACCT TAGGCCTTCC TCTCTCCAGA TGTTTCCAGA CTCCTTGAG ACACGGAGCC	1200
CAGCCCTCCC CATGGAGCCA GCTCCCTCTA TTTATGTTTG CACTTGTGAT TATTTATTAT	1260
TTATTTATTA TTTATTTATT TACAGATGAA TGTATTTATT TGGGAGACCG GGGTATCCTG	1320
GGGGACCCAA TGTAGGAGCT GCCTTGGCTC AGACATGTTT TCCGTGAAAA CGGAGGCTGA	1380
ACAATAGGCT GTTCCCATGT AGCCCCCTGG CCTCTGTGCC TTCTTTTGAT TATGTTTTTT	1440
AAAATATTAT CTGATTAAGT TGTCTAAACA ATGCTGATTT GGTGACCAAC TGTCACCTCAT	1500

TGCTGAGGCC TCTGCTCCCC AGGGAGTTGT GTCTGTAATC GGCCTACTAT TCAGTGGCGA 1560
GAAATAAAGG TTGCTTAGGA AAGAA 1585

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGGAATTTCA TGCCCCAGTT GACAACATAG 30

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CACTCGAGAA CTTTTTCTCC TTAGATCAT ACAA 34

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGGGAATTCA TGCCCCAGTT GACAACATAG 30

(2) INFORMATION FOR SEQ ID NO: 16:

78

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAGCTCGAGT GTCACAGACT TCTTTTCCA

29

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AAACGAATTC CATCCAGTAA GTCCATTACT

30

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GAGCTCGAGT GTCACAGACT TCTTTC

26

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCGGCCGCGC ATGTACAGCA TGCAGCTCGC A

31

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCGGCCGCTA AATAAATAGA GAGCCTTATG

30

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1011 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGTTTATTTT CCAGATGCAA TCAATGCCCC AGTCACCTGC TGTTATAACT TCACCAATAG	60
GAAGATCTCA GTGCAGAGGC TCGCGAGCTA TAGAAGAATC ACCAGCAGCA AGTGTCCCAA	120
ACAAGCTGTG ATGTGAGTTC AGCACACCAA CCTTCCCTGG CCTGAAGTTC TTCCTTGTGG	180
AGCAAGGGAC AAGCCTCATA AACCTAGAGT CAGAGAGTGC ACTATTTAAC TTAATGTACA	240
AAGGTTCCCA ATGGGAAAAC TGAGGCACCA AGGGAAAAAG TGAACCCCAA CATCACTCTC	300
CACCTGGGTG CCTATTCAGA ACACCCAATT TCTTTAGCTT GAAGTCAGGA TGGCTCCACC	360
TGGACACCTA TAGGAGCAGT TTGCCCTGGG TTCCCTCCTT CCACCTGCGT TCCTCCTCTA	420
GCTCCCATGG CAGCCCTTTG GTGCAGAATG GGCTGCACTT CTAGACCAAA ACTGCAAAGG	480

AAC TTCATCT AACTCTGTCC TCCCTCCCA CAGCTTACAG ACCATTGTGG CAAGGAGATC	540
TGTGCTGACC CCAAGCAGAA GTGGGTTTCAG GATTCCATGG ACCACCTGGA CAAGCAAACC	600
CAAAC TCCGA AGACTTGAAC ACTCACTCCA CAACCCAAGA ATCTGCAGCT AACTTATTTT	660
TCCCTAGCTT TCCCCAGACA CCTTGTTTAT TTTATTATAA TGAATTTTGT TTGTTGATGT	720
GAAACATTAT GCCTTAAGTA ATGTTAATTC TTATTTAAGT TATTGATGTT TTAAGTTTAT	780
CTTTCATGGT ACTAGTGTTT TTTAGATACA GAGACTTGGG GAAATTGCTT TTCCTCTTGA	840
ACCACAGTTC TACCCCTGGG ATGTTTTGAG GGTCTTTGCA AGAATCATT ATACAAAGAA	900
TTTTTTTAA CATTCCAATG CATTGCTAAA ATATTATTGT GGAAATGAAT ATTTTGTAAC	960
TATTACACCA AATAAATATA TTTTGTACA AAAAAAAAAA AAAAAAAAAA A	1011

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3194 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AAGCTTGCTG AGAGTGGCTG CAGTCTCGCT GCTGGATGTG CACATGGTGG TCATTCCCTC	60
TGCTCACAGG GGCAGGGGTC CCCCCTTACT GGACTCAGGT TGCCCCCTGC TCCAGGTCCT	120
GGGTGGGAGC CCATGTGAAC TGTCAGTGGG GCAGGTCTGT GAGAGCTCCC CTCACACTCA	180
AGTCTCTCAC AGTGGCCAGA GAAGAGGAAG GCTGGAGTCA GAATGAGGCA CCAGGGCGGG	240
CATAGCCTGC CCAAAGGCCC CTGGGATTAC AGGCAGGATG GGGAGCCCTA TCTAAGTGTC	300
TCCCACGCCC CACCCAGCC ATTCCAGGCC AGGAAGTCCA AACTGTGCCC CTCAGAGGGA	360
GGGGGCAGCC TCAGGCCCAT TCAGACTGCC CAGGGAGGGC TGGAGAGCCC TCAGGAAGGC	420
GGGTGGGTGG GCTGTGGTT CTGGAAAGG TTCATTAATG AAAACCCCCA AGCCTGACCA	480
CCTAGGGAAG AGGCTCACCG TTCCCATGTG TGGCTGATAA GGGCCAGGAG ATTCCACAGT	540
TCAGGTAGTT CCCCCGCTC CTGGCATT TGTGGTCACC ATTAATCATT TCCTCTGTGT	600
ATTTAAGAGC TCTTTTGCCA GTGAGCCCAG TACACAGAGA GAAAGGCTAA AGTTCTCTGG	660
AGGATGTGGC TGCAGAGCCT GCTGCTCTTG GGCAGTGTGG CCTGCAGCAT CTCTGCACCC	720
GCCCCGCTGC CCAGCCCCAG CACGCAGCCC TGGGAGCATG TGAATGCCAT CCAGGAGGCC	780
CGGCGTCTCC TGAACCTGAG TAGAGACACT GCTGCTGAGA TGGTAAGTGA GAGAATGTGG	840
GCCTGTGCCT AGGCCACCCA GCTGGCCCT GACTGGCCAC GCCTGTCAGC TTGATAACAT	900

GACATTTTCC TTTTCTACAG AATGAAACAG TAGAAGTCAT CTCAGAAATG TTTGACCTCC	960
AGGTAAGATG CTTCTCTCTG ACATAGCTTT CCAGAAGCCC CTGCCCTGGG GTGGAGGTGG	1020
GGACTCCATT TTAGATGGCA CCACACAGGG TTGTCCACTT TCTCTCCAGT CAGCTGGCTG	1080
CAGGAGGAGG GGGTAGCAAC TGGGTGCTCA AGAGGCTGCT GGGCGTGCCC CTATGGCAGT	1140
CACATGAGCT CCTTTATCAG CTGAGCGGCC ATGGGCAGAC CTAGCATTCA ATGGCCAGGA	1200
GTCACCAGGG GACAGGTGGT AAAGTGGGGG TCACTTCATG AGACAGGAGC TGTGGGTTTG	1260
GGGCGCTCAC TGTGCCCCGA GACCAAGTCC TGTGAGACA GTGCTGACTA CAGAGAGGCA	1320
CAGAGGGGTT TCAGGAACAA CCCTTGCCCA CCCAGCAGGT CCAGGTGAGG CCCCACCCCC	1380
CTCTCCCTGA ATGATGGGGT GAGAGTCACC TCCTTCCCTA AGGCTGGGCT CCTCTCCAGG	1440
TGCGCTGAG GGTGGCCTGG GCGGGGCACT GAGAAGGGCA GGTTCGTGCC TGCCATGGAC	1500
AGGGCAGGGT CTATGACTGG ACCCAGCTG TGGCCCTCCC AAGCCCTACT CCTGGGGGCT	1560
GGGGGCAGCA GCAAAAAGGA GTGGTGGAGA GTTCTGTAC CACTGTGGGC ACTTGGCCAC	1620
TGCTCACC GAACGACAT TTTCCACAGG AGCCGACCTG CCTACAGACG CGCCTGGAGC	1680
TGTACAAGCA GGGCCTGCGG GGCAGCCTCA CCAAGCTCAA GGGCCCCTTG ACCATGATGG	1740
CCAGCCACTA CAAGCAGCAC TGCCCTCCAA CCCCAGTGAG TGCCTACGGC AGGGCCTCCA	1800
GCAGGAATGT CTTAATCTAG GGGGTGGGGT CGACATGGGG AGAGATCTAT GGCTGTGGCT	1860
GTTCAGGACC CCAGGGGGTT TCTGTGCCAA CAGTTATGTA ATGATTAGCC CTCCAGAGAG	1920
GAGGCAGACA GCCCATTTC TCCCAAGGAG TCAGAGCCAC AGAGCGCTGA AGCCCACAGT	1980
GCTCCCCAGC AGGAGCTGCT CCTATCCTGG TCATTATTGT CATTACGGTT AATGAGGTCA	2040
GAGGTGAGGG CAAACCCAAG GAACTTGGG GCCTGCCCAA GGCCCAGAGG AAGTGCCAG	2100
GCCCAAGTGC CACCTTCTGG CAGGACTTTC CTCTGGCCCC ACATGGGGTG CTTGAATTGC	2160
AGAGGATCAA GGAAGGGAGG CTA CTGGA TGGACAAGGA CCTCAGGCAC TCCTTCTGCG	2220
GGAAGGGAGC AAAGTTTGTG GCCTGACTC CACTCCTTCT GGGTGCCAG AGACGACCTC	2280
AGCCCAGCTG CCCTGCTCTG CCCTGGGACC AAAAAGGCAG GCGTTTGA CT GCCCAGAAGG	2340
CCAACCTCAG GCTGGCACTT AAGTCAGGCC CTTGACTCTG GCTGCCACTG GCAGAGCTAT	2400
GCACTCCTTG GGAACACGT GGGTGGCAGC AGCGTCACCT GACCCAGGTC AGTGGGTGTG	2460
TCCTGGAGTG GGCCTCCTGG CCTCTGAGTT CTAAGAGGCA GTAGAGAAAC ATGCTGGTGC	2520
TTCCTTCCCC CACGTTACCC ACTTGCCCTGG ACTCAAGTGT TTTTATTTT TCTTTTTTTA	2580
AAGGAAACTT CCTGTGCAAC CCAGATTATC ACCTTTGAAA GTTTCAAAGA GAACCTGAAG	2640
GACTTCTGCT TTGTCATCCC CTTTGA CTG TGGGAGCCAG TCCAGGAGTG AGACCGGCCA	2700
GATGAGGCTG GCCAAGCCGG GGAGCTGCTC TCTCATGAAA CAAGAGCTAG AAACCTCAGGA	2760
TGGTCATCTT GGAGGGACCA AGGGGTGGGC CACAGCCATG GTGGGAGTGG CCTGGACCTG	2820
CCCTGGGCCA CACTGACCCT GATACAGGCA TGGCAGAAGA ATGGGAATAT TTTATACTGA	2880

CAGAAATCAG TAATATTTAT ATATTTATAT TTTTAAATA TTTATTTATT TATTTATTTA	2940
AGTTCATATT CCATATTTAT TCAAGATGTT TTACCGTAAT AATTATTATT AAAAATATGC	3000
TTCTACTTGT CCAGTGTCTT AGTTTGT TTTT TAACCATGAG CAAATGCCAG TGGGTGCCTG	3060
CCTTCCCATG AGGCAGGGGA GGCAGGAAAC GGGGAGGTGG AGAGGGGGCG GGGGCCTCCC	3120
AGGCGTTGGG CACTATCCAA GGGCCAACAC TGTCAGAGCA GAGGGGAGGT GAGAGCCGGG	3180
CATAGTCGGA ATTC	3194

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1491 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CCAAAGAAAA AGTGATTGT CATTGCTTTA TAGACTGTAA GAAGAGAACA TCTCAGAAGT	60
GGAGTCTTAC CCTGAAATCA AAGGATTTAA AGAAAAAGTG GAATTTTTCT TCAGCAAGCT	120
GTGAAACTAA ATCCACAACC TTTGGAGACC CAGGAACACC CTCCAATCTC TGTGTGTTTT	180
GTAAACATCA CTGGAGGGTC TTCTACGTGA GCAATTGGAT TGTCATCAGC CCTGCCTGTT	240
TTGCACCTGG GAAGTGCCTT GGTCTTACTT GGTGCCAAT TGTTGGCTTT CACTTTTGAC	300
CCTAAGCATC TGAAGCCATG GGCCACACAC GGAGGCAGGG AACATCACCA TCCAAGTGTC	360
CATACCTCAA TTTCTTTCAG CTCTTGGTGC TGGCTGGTCT TTCTCACTTC TGTTCAAGTG	420
TTATCCACGT GACCAAGGAA GTGAAAGAAG TGGCAACGCT GTCCTGTGGT CACAATGTTT	480
CTGTTGAAGA GCTGGCACA ACTCGCATCT ACTGGCAAAA GGAGAAGAAA ATGGTGCTGA	540
CTATGATGTC TGGGGACATG AATATATGGC CCGAGTACAA GAACCGGACC ATCTTTGATA	600
TCACTAATAA CCTCTCCATT GTGATCCTGG CTCTGCGCCC ATCTGACGAG GGCACATACG	660
AGTGTGTTGT TCTGAAGTAT GAAAAGACG CTTTCAAGCG GGAACACCTG GCTGAAGTGA	720
CGTTATCAGT CAAAGCTGAC TTCCCTACAC CTAGTATATC TGACTTTGAA ATTCCAACCT	780
CTAATATTAG AAGGATAATT TGCTCAACCT CTGGAGGTTT TCCAGAGCCT CACCTCTCCT	840
GGTTGGAAAA TGGAGAAGAA TTAAATGCCA TCAACACAAC AGTTTCCCAA GATCCTGAAA	900
CTGAGCTCTA TGCTGTTAGC AGCAAACTGG ATTTCAATAT GACAACCAAC CACAGCTTCA	960
TGTGTCTCAT CAAGTATGGA CATTTAAGAG TGAATCAGAC CTTCAACTGG AATACAACCA	1020
AGCAAGAGCA TTTTCTGAT AACCTGCTCC CATCCTGGGC CATTACCTTA ATCTCAGTAA	1080
ATGGAATTTT TGTGATATGC TGCCTGACCT ACTGCTTTGC CCCAAGATGC AGAGAGAGAA	1140

GGAGGAATGA	GAGATTGAGA	AGGGAAAGTG	TACGCCCTGT	ATAACAGTGT	CCGCAGAAGC	1200
AAGGGGCTGA	AAAGATCTGA	AGGTAGCCTC	CGTCATCTCT	TCTGGGATAC	ATGGATCGTG	1260
GGGATCATGA	GGCATTCTTC	CCTTAACAAA	TTTAAGCTGT	TTTACCCACT	ACCTCACCTT	1320
CTTAAAAACC	TCTTTCAGAT	TAAGCTGAAC	AGTTACAAGA	TGGCTGGCAT	CCCTCTCCTT	1380
TCTCCCCATA	TGCAATTTC	TTAATGTAAC	CTCTTCTTTT	GCCATGTTTC	CATTCTGCCA	1440
TCTTGAATTG	TCTTGTGAGC	CAATTCATTA	TCTATTAAAC	ACTAATTGGA	G	1491

CLAIMS

1. A DNA construct comprising (i) means for expression of a coding
sequence in a tumour cell and (ii) a said coding sequence
encoding a cytokine.
2. A construct according to Claim 1 wherein the said means for
expression provides for specific expression selectively in tumour
cells.
3. A construct according to Claim 2 wherein the tumour cells are
melanoma cells.
4. A construct according to Claim 2 wherein the tumour cells are
breast tumour cells.
5. A construct according to Claim 2 wherein the tumour cells are
colon tumour cells.
6. A construct according to Claim 2 wherein the tumour cells are
pancreatic tumour cells.
7. A construct according to Claim 2 wherein the tumour cells are
prostate tumour cells.
8. A construct according to Claim 3 wherein the said means for
expression is a promoter or an analogue or part thereof forming
part of a gene expressed exclusively in the melanin synthesis
pathway.

9. A construct according to Claim 8 wherein the gene is tyrosinase or TRP-1.
- 5 10. A construct according to Claim 4 wherein the said means for expression is provided by the *c-erb-B2* gene promoter or the MUC1 gene promoter or the *c-erb-B3* gene promoter.
11. A construct according to Claim 5 wherein the said means for expression is provided by the CEA gene promoter.
- 10 12. A construct according to Claim 6 wherein the said means for expression is provided by the MUC1 gene promoter.
- 15 13. A construct according to Claim 7 wherein the said means for expression is provided by the PSA gene promoter.
14. A construct according to any one of the preceding claims wherein the cytokine is interleukin-2 or interleukin-4.
- 20 15. A construct according to any one of the preceding claims further comprising a B7 coding sequence and means for expression thereof in a tumour cell.
- 25 16. A composition comprising a construct according to any one of the preceding claims and means for selectively delivering it to a tumour.
- 30 17. A composition according to Claim 16 wherein the selective delivery means is a liposome carrying tumour cell targeting means or a retrovirus or adenovirus specific for the tumour cells.

18. A method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a construct according to any one of Claims 1 to 15.
- 5 19. A method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a construct according to any one of Claims 1 to 15 expressing at least two coding sequences encoding respective cytokines wherein the said cytokines may be the same as or different from one
10 another.
20. A method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a plurality of constructs according to any one of Claims 1 to 15
15 expressing at least two coding sequences encoding respective cytokines wherein the said cytokines may be the same as or different from one another.
21. A method according to Claims 19 or 20 wherein the cytokines are
20 chosen from interleukin-2, interleukin-4, macrophage colony stimulating factor, interferon- γ , tumour necrosis factor and interleukin-7.
22. A method according to Claims 19 or 20 wherein the coding
25 sequences encode interleukin-2, interleukin-4 and macrophage colony stimulating factor and are present in 1:1:1 molar ratio.
23. A method according to any one of Claims 18 to 20 wherein the
30 tumour cells are melanoma, breast, pancreas, prostate or colon cells and naked DNA is injected directly into the tumour.

24. A method according to any one of Claims 18 to 23 additionally comprising administering a chemotherapeutic agent.
- 5 25. A method according to Claim 24 wherein the chemotherapeutic agent is at least one of cisplatin, dacarbazine, tamoxifen, nitrosourea, vinca alkaloid, melphalan, doxorubicin, adriamycin, etoposide and 5-fluorouracil.
- 10 26. A method according to any one of Claims 18 to 25 further comprising delivering into cells of the tumour a construct comprising a B7 coding region and means for expression thereof in a tumour cell.
- 15 27. A method according to any one of Claims 18 to 25 comprising delivering into cells of the tumour a construct comprising a B7 coding region and a cytokine coding region and means for expression thereof in a tumour cell.

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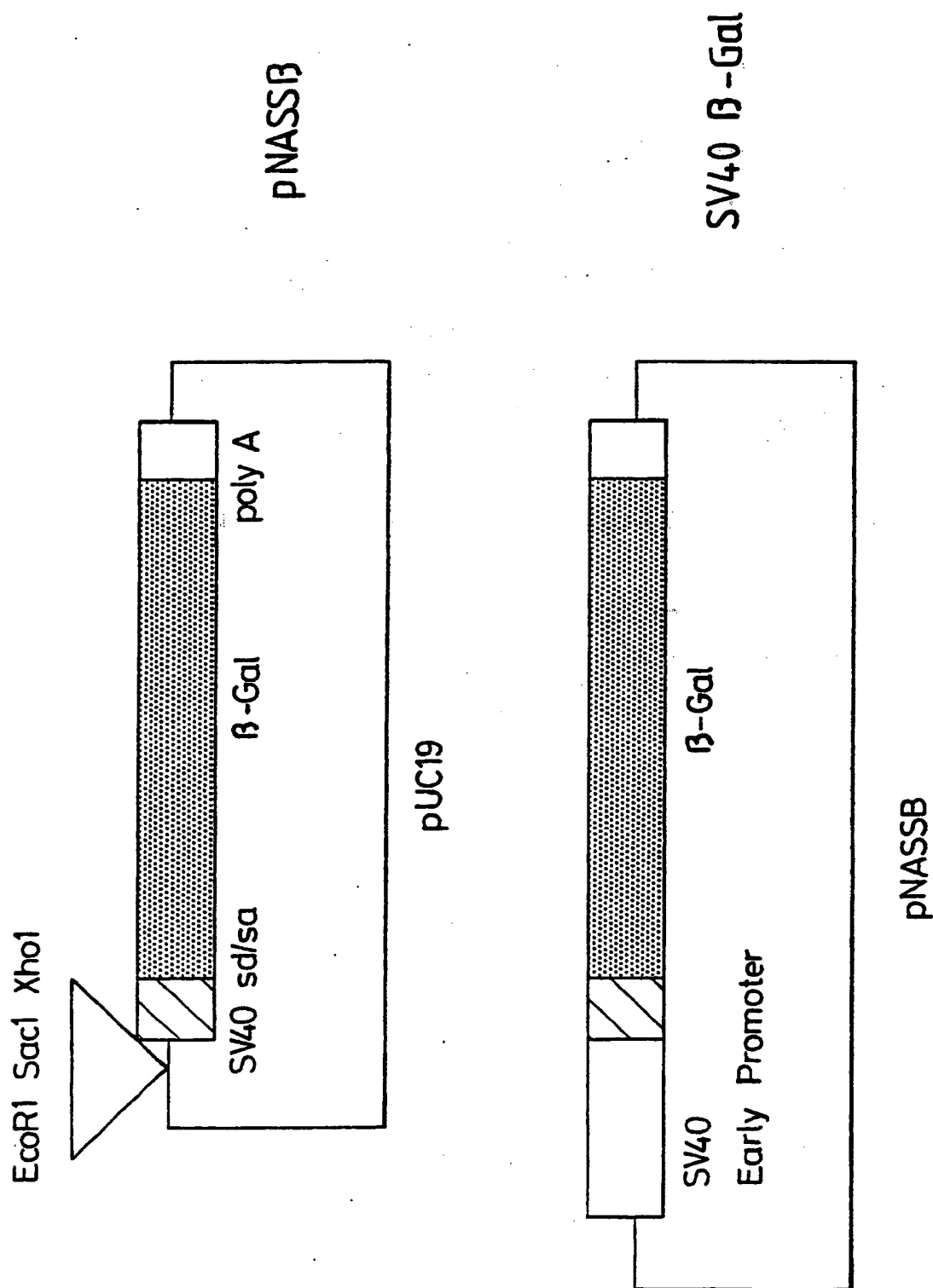


Fig. 1 (PAGE 1 of 3)

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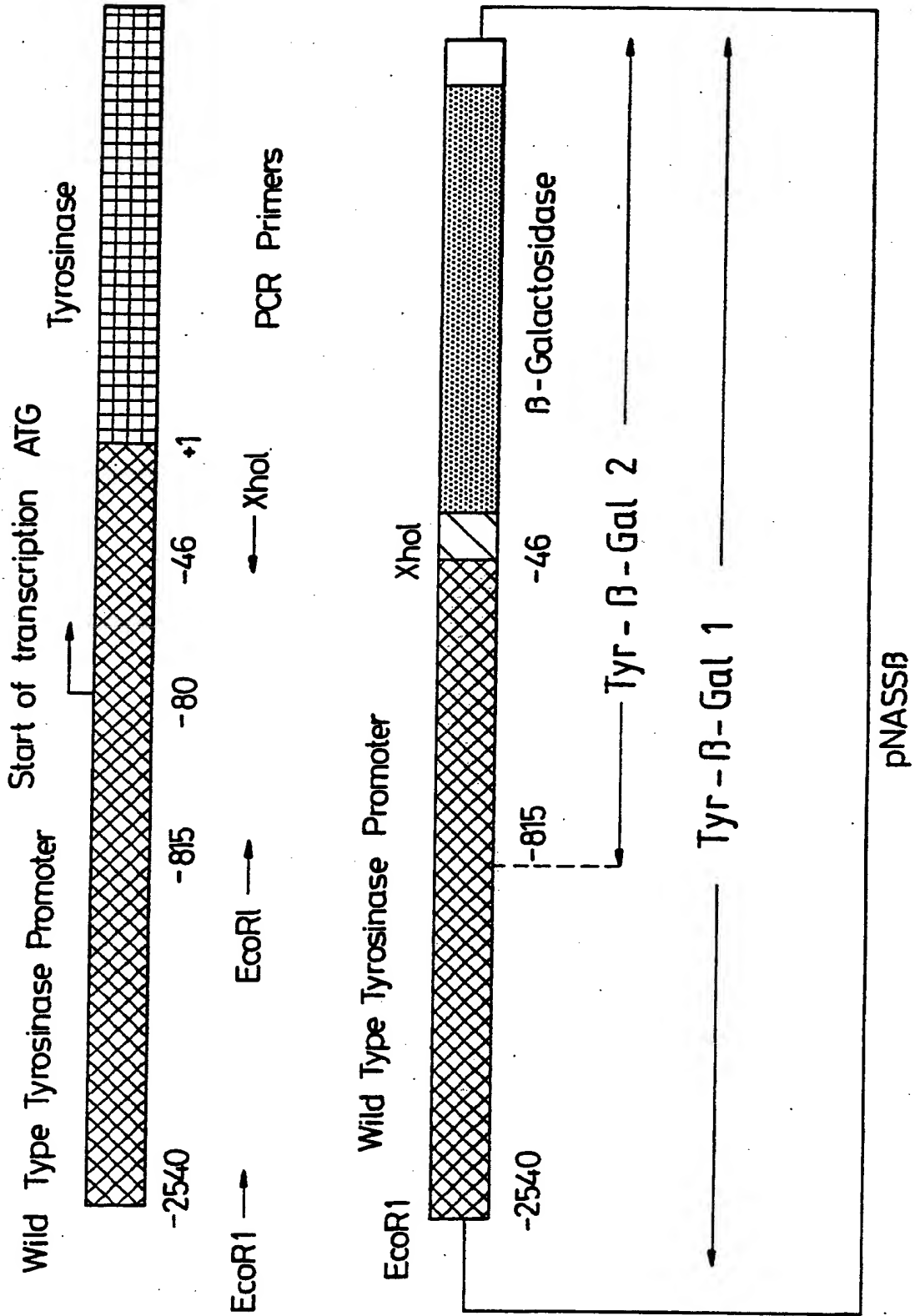


Fig. 1 (PAGE 2 of 3)

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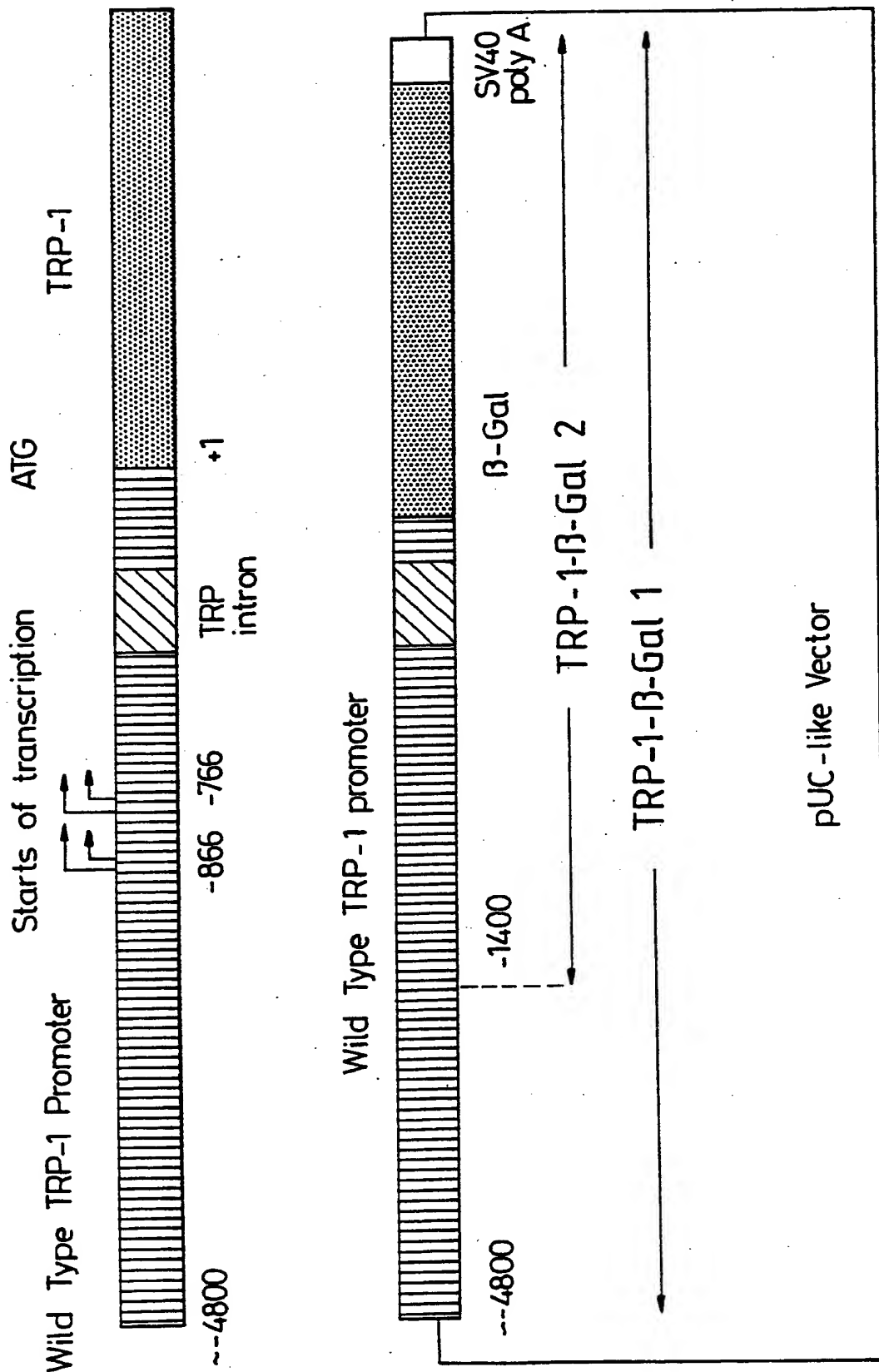
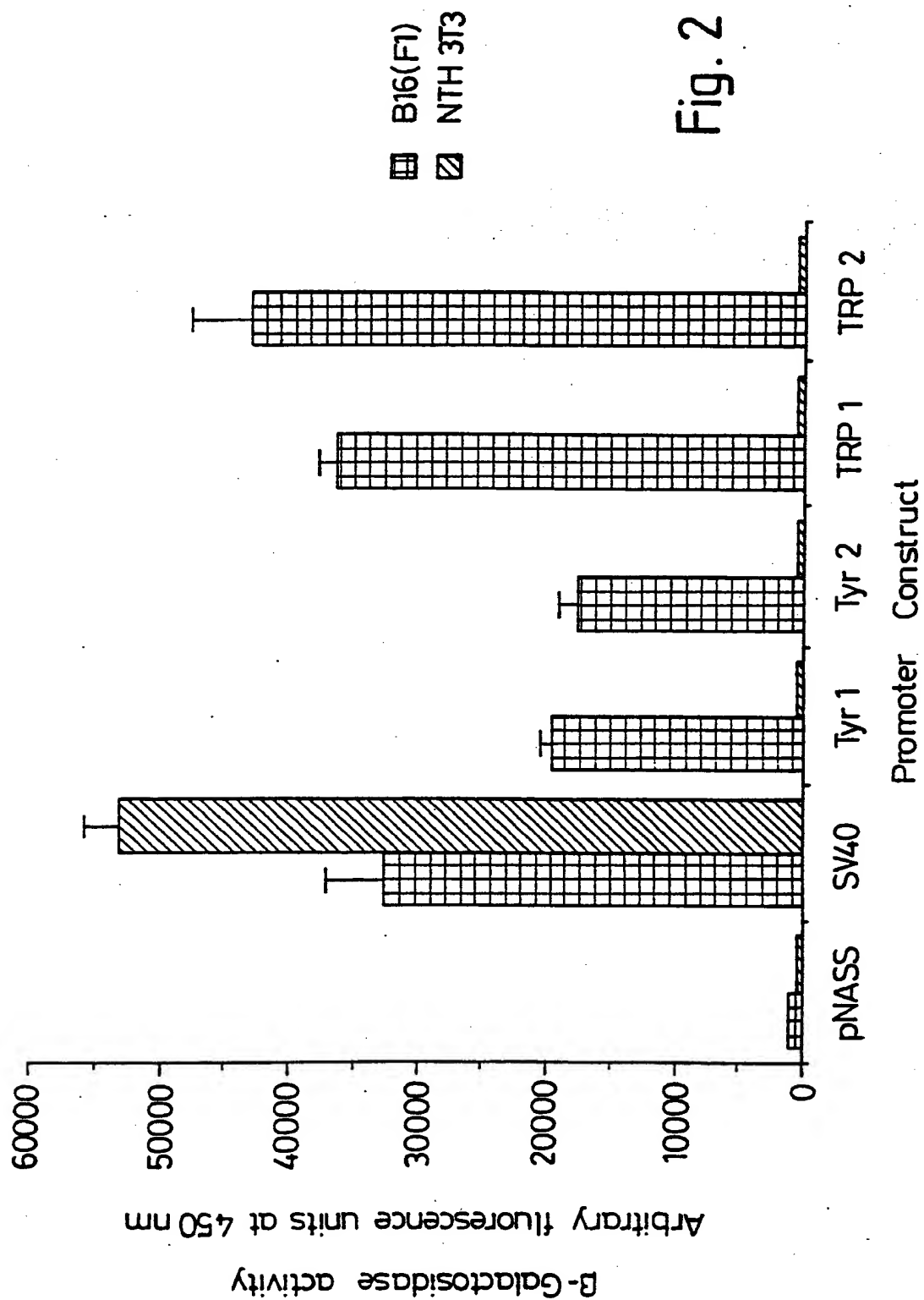
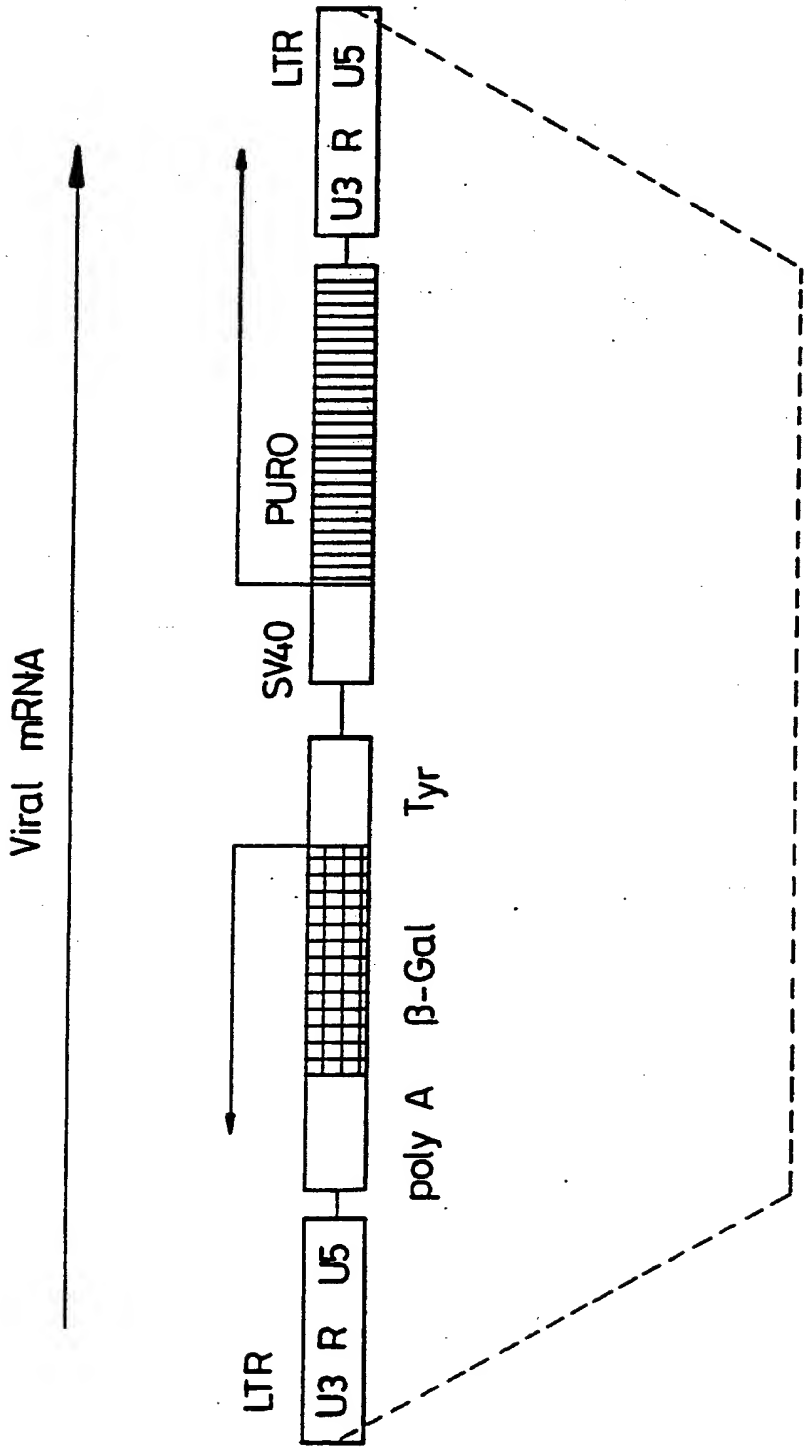


Fig. 1 (PAGE 3 of 3)

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pBabe Puro (Tyr- β -Gal)

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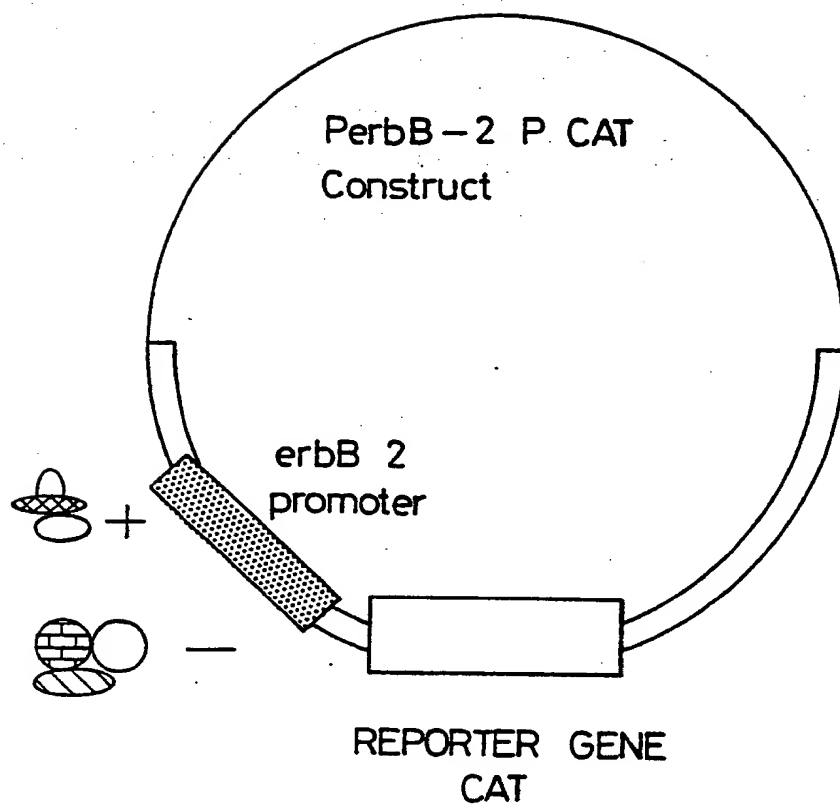


Fig. 4

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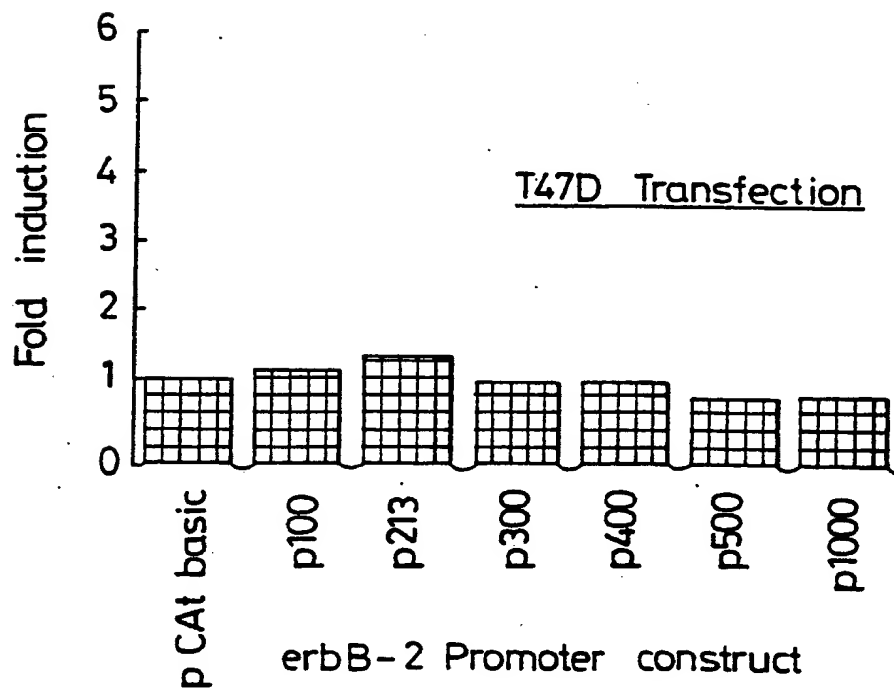
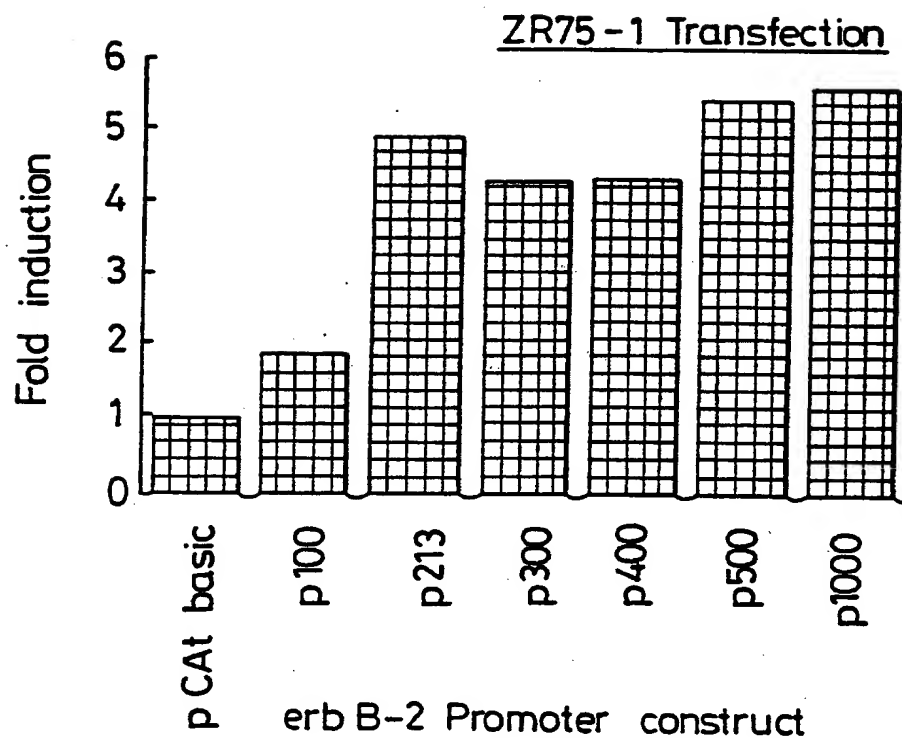


Fig. 5

Fig. 6

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NcoI
 ↓
 -1571 CATGGTGTCCGACTTATGCCCCGAGAAGATGTTGAGCAAACTTATCGCTTATCTGCTTCTCATAGAGTCTT
 -1501 GCAGACAAACTGCGCAACTCGTGAAGGTAGGGGATCTGGTGGACCTGCAGGTCAACGGATCCCTTCT
 -1431 TGACCAAGTATAGCTGCATTCTTGGCTGGGGCATTCCAACTAGAACTGCCAAATTAGCACATAAAATAA
 -1361 GGAGGCCCCAGTTAAATTGAATTTTCAGATAAACAAATGAATAAATTGTTAGTATAAAATATGTCCCATGCAA
 -1291 TATCTTGTGAAATTAAAAAAAAGTCTTCTTCCATGCCCCACCCCTACCACTAGGCCCTAAGGAATAG
 -1221 GGTCAAGGGCTCCAAATAGAAATGTGTTGAGAAGTGAATTAAGCAGGCTAATAGAGGCAAGGGGCAAA
 -1151 GAAGAAACCTTGAATGCATTGGGTGCTGGTGCTCCTTAAATAAGCAAGAGGTGCATTTTGAAGAAT
 -1081 TGAGATAGAACTCTTTTGGGCTGGTGCTGCTCGTGGTTGTAATTCAGCACTTTGGGAGGCTGAG
 -1011 GCGGGAGGATCACCTGAGGTTGGGAGTTCAAGACCAGCCTCAACCACTGGAGAACCTGTCTTTACTAA
 -941 AAATACAAAAAATTCAGCTGGTCAATGGTGGCACATGCCCTGTAATCCAGCTGCTCGGGAGGCTGAGGCAG
 -871 GAGAACTCACTTGAACCCAGGGAGGAGGTTGTGGTGAGCAGAGATCGGCCATTGCTCTCCAGCCTGGG
 -801 CAACAAGAGCAAAAGTTCGTTTAAAAAAAAGTCTTTCGATGTGACTGTCTCTCCCAAATTG
 -731 TAGACCTCTTAAGATCATGCTTTTCAGATACTTCAAAGATTCCAGAAGATATGCCCGGGGTCCTGGA
 -661 AGCCCAAGGTAAACAACAACATCCCCCTCCTTGACTATCAATTTTACTAGAGGATGTGGTGGGAAAC
 -591 CATTATTTGATATTAAAACAAATAGGCTTGGATGGAGTAGGATGCAAGCTCCCCAGGAAAGTTTAAGAT
 -521 AAAACCTGAGACTTAAAGGGTGTAAAGAGTGGCAGCCTAGGGAATTTATCCCGGACTCCGGGGGAGGGG
 -451 GCAGAGTCACCAGCCTCTGCATTAGGGATTCTCCGAGGAAAGTGTGAGAACGGCTGCAGGCAACCCAG

FIG. 7 (PAGE 1 OF 2)

-381 GCGTCCCGCGCTAGGAGGGACGACCCAGGCCTGCCCGAAGAGAGGAGAAAGTGAAGCTGGGAGTTGCC
 -311 GACTCCAGACTTCGTTGGAATGCAGTTGGAGGGGGCGAGCTGGGAGCGCGCTTGCTCCCAATCACAGGA
 -241 GAAGGAGGAGGTGGAGGAGGGCTGCTTGAGGAAGTATAAAGTGTGTGAAGCTGAGATTCCCC
 -171 TCCATTGGGACCGGAGAAACCAGGGGAGCCCCCGGGCAGCGCGCGCCCTTCCCACGGGGCCCTTTAC
 -101 TGCGCGCGCGCGCGCCCCACCCCTCGCAGCACCCCGCGCCCCCGCCCTCCAGCCGGGTCCAGCCG
 -31 GAGCCATGGGGCGGAGCGGCAGTGAGCACCATGGAGCTGG
 -1 NcoI

FIG. 7 (PAGE 2 OF 2)

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Fig. 8 (PAGE 1 of 3)

AGTGGGGAGCCCCCTCCCGGGTAGAAGTGGAAAGCGGGCTCCGGGTCTGTTCACGGCTGGAACC
351 420

Sma I

ACCCCGCCCCCATCCAAATCCCCGGGAGAGGCCCGCGCGCGGGTCTGGAGGAGGAAGCGGCCAG
421 490

AGACAGTGCAATTTACGCGGTCTCTGTGGCTCCGGTTCCTGGGTGGTGATGAATTATGGGGTTTCG
491 560

AGTCTGGGAGAACTGAGGTGGCCTGGACGTGAGGCAAAACACCCCTCCCCCTCAAAACACACAGAGA
561 630

FP/D

GAAATATTCACATTCTGAGAGAAATCCACCAGTGAACCAACCGGCTAGGGGAGTTGAGTGATTGGGTT
631 700

FP/E

AATGGGCGAGGCCAACTTTCAGGGGCGAGGGCTTTGGAGAGCTTTCCACTCCCTCATTTACCCCTTCC
701 770

Pst I

CTGGATCTGGGGGCTTTCGGAATCTCGACCTCCCCTTGGCCTATCTCCTGCAGAAAAATTAGGGTGAGCC
771 840

CCATCCTCGATCTGCTCCGCCAAGTTGCGGGACCGCGGGCGTGGCACGCTCAGGGGCGAGCGGTCCGAG
841 910

Fig. 8 (PAGE 2 of 3)

GCTCCGCAATCCCCACTCCAGCCTCGCGCGGAGGGCGCGCGCGGTGACTACACCCCTTCCCCTCTG
 911 980
 ↑ ↑ ↑
 CGTTCCCTCCCTCTCTCTCTCTCTCACACACACACCCCTCCCCCTGCCATCCCTCCCCCGACTC
 981 P 1050
 ↑
 CGGCTCCGGCTCCGATTGCAATTTGCAACCTCCGCTGCCGTGCCGCGCAGCACCACCAATTGCCAGCGG
 1051 +1 K 1120
 ↑
 TTCAGGTGGCTCTTGCCCTCGATGTCCTAGCCTAGGGGCCCCCGGGCGGACTTGCGTGGGCTCCCTTCAC
 1121 SmaI 1190
 PstI SmaI
 CCTCTGCGGAGTCATGAGGGCGGAACGACGCTCTGCAGGTGCTGGGCTTGCTTTTACGCTGGCCCGGGC
 11191 METArgAlaAsnAspAlaLeuGlnValLeuGlyLeuLeuPheSerLeuAlaArgGly
 1260
 1st intron
 TCCGAGGTGGGCAACTCTCAGGCAGGTAAGTGCCCAGAGACACC
 1305
 SerGluValGlyAsnSerGlnAla
 1261

Fig. 8 (PAGE 3 of 3)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01730

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 A61K48/00 C12N15/86 C12N15/26 C12N15/85 A61K33/24
 A61K31/70 A61K31/71 //(A61K33/24, 31:71, 31:70, 31:505, 31:475,
 31:415, 31:195, 31:17, 31:135), C12N15/24, C12N15/27, C12N15/28,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>CANCER RESEARCH vol. 53 , 1 March 1993 pages 962 - 967 R. G. VILE ET AL 'In vitro and in vivo targeting of gene expression to melanoma cells' see the whole document see especially page 966 right column</p> <p style="text-align: center;">--- -/--</p>	<p>1-3, 8, 9, 16-18, 23</p>

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* Special categories of cited documents :

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Date of the actual completion of the international search

30 November 1993

Date of mailing of the international search report

16. 12. 93

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
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 Fax (+31-70) 340-3016

Authorized officer

Le Cornec, N

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/GB 93/01730

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH. vol. 19, no. 14, 1991, ARLINGTON, VIRGINIA US pages 3799 - 3804 I. J. JACKSON ET AL 'The Tyrosinase-related protein-1 gene has a structure and promoter sequence very different from Tyrosinase' cited in the application see the whole document especially the abstract, page 3802 left column line 9 -right column line 4, page 3803 right column --- -/--	1-3,8,9, 16-18,23

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

30 November 1993

Date of mailing of the international search report

3. 12. 93

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

Le Cornec, N

INTERNATIONAL SEARCH REPORT

Intern. Appl. No.
PCT/GB 93/01730

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88 , September 1991 , WASHINGTON US pages 8039 - 8043 B. E. HUBER ET AL 'Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma : A innovative approach for cancer therapy' see the whole document especially page 8039</p> <p>---</p>	1-3,8,9, 16-18,23
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 88 , January 1991 , WASHINGTON US pages 164 - 168 M. BRADL ET AL 'Malignant melanoma in transgenic mice' cited in the application see page 164 see page 167, right column, line 21 - line 39</p> <p>---</p>	1-3,8,9, 16-18,23
A	<p>ANNALS OF PASTIC SURGERY vol. 28, no. 1 , January 1992 pages 114 - 118 M. SIVANANDHAM ET AL 'Prospects for gene therapy and lymphokine therapy for metastatic melanoma'</p> <p>---</p>	
X	<p>CELL vol. 60, no. 3 , 9 February 1990 , CAMBRIDGE, MA US pages 397 - 403 E. R. FEARON ET AL 'Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response' cited in the application see the whole document especially page 400 right column, table 2 and pages 401-402</p> <p>---</p>	1,14, 18-21, 234
Y	<p>EP,A,0 415 731 (THE WELLCOME FOUNDATION LIMITED) 6 March 1991 see the whole document</p> <p>---</p>	1-3,8,9, 16-18
X	<p>JOURNAL OF IMMUNOLOGY vol. 146, no. 9 , 1 May 1991 , BALTIMORE US pages 3227 - 3234 A.L. ASHER ET AL 'Murine tumor cells transduced with the gene for tumor necrosis factor-alpha' see the whole document</p> <p>---</p>	1,18
	<p>---</p> <p>-/--</p>	

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/GB 93/01730

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGY TODAY vol. 11, no. 6 , 1990 , CAMBRIDGE GB pages 196 - 200 S.J. RUSSEL 'Lymphokine gene therapy for cancer' see the whole document ---	1,16
T	NATURE vol. 357 , 11 June 1992 , LONDON GB pages 455 - 460 A. DUSTY MILLER ET AL 'Human gene therapy comes of age' -----	

INTERNATIONAL SEARCH REPORT

International application No.

PLT/GB93/01730

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 18-27 (as far as they concern in vivo methods) are directed to the treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Information on patent family members

PCT/GB 93/01730

Form PCT/ISA/210 (patent family annex) (July 1992)

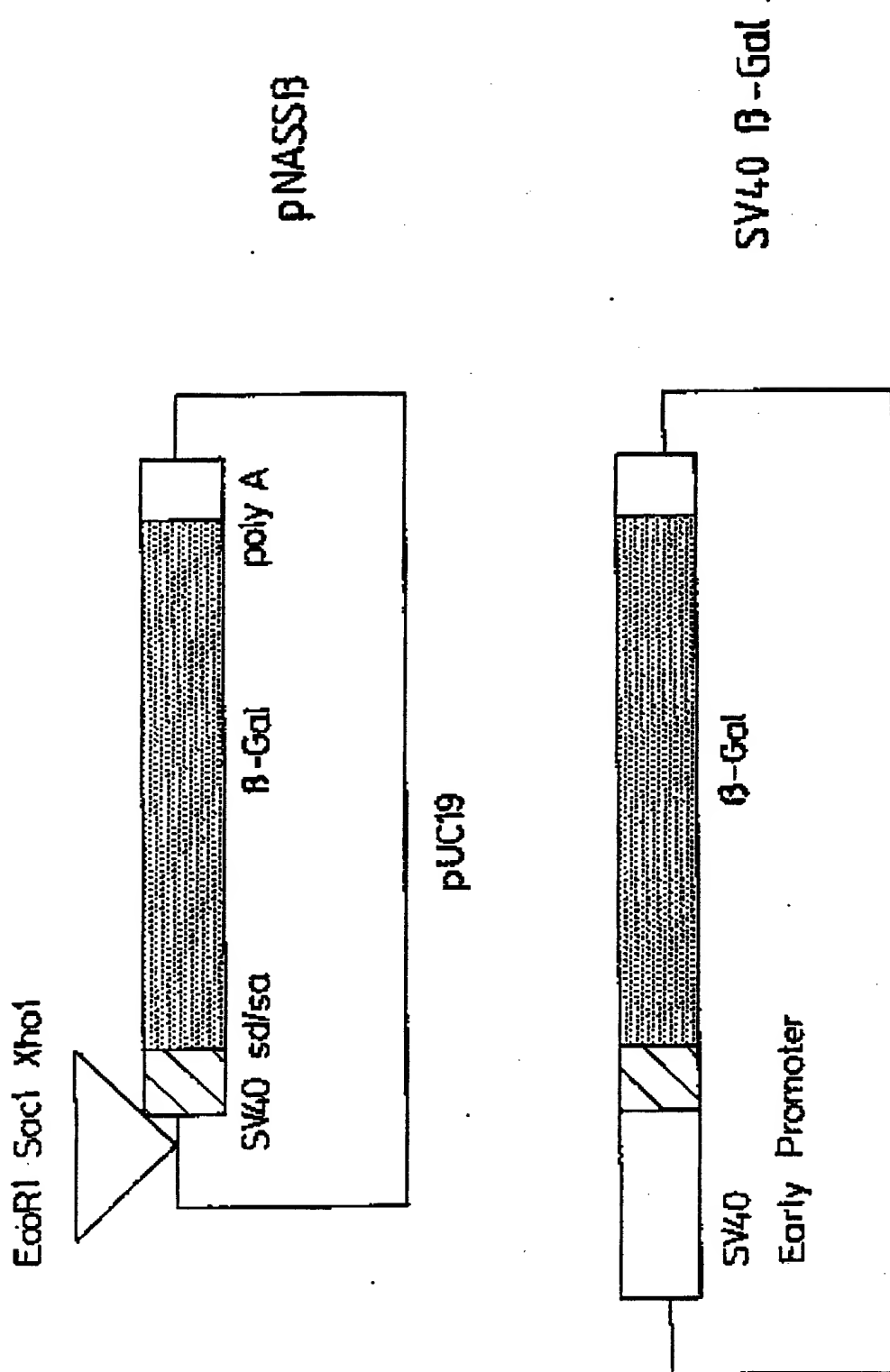


Fig. 1 (PAGE 1 of 3)

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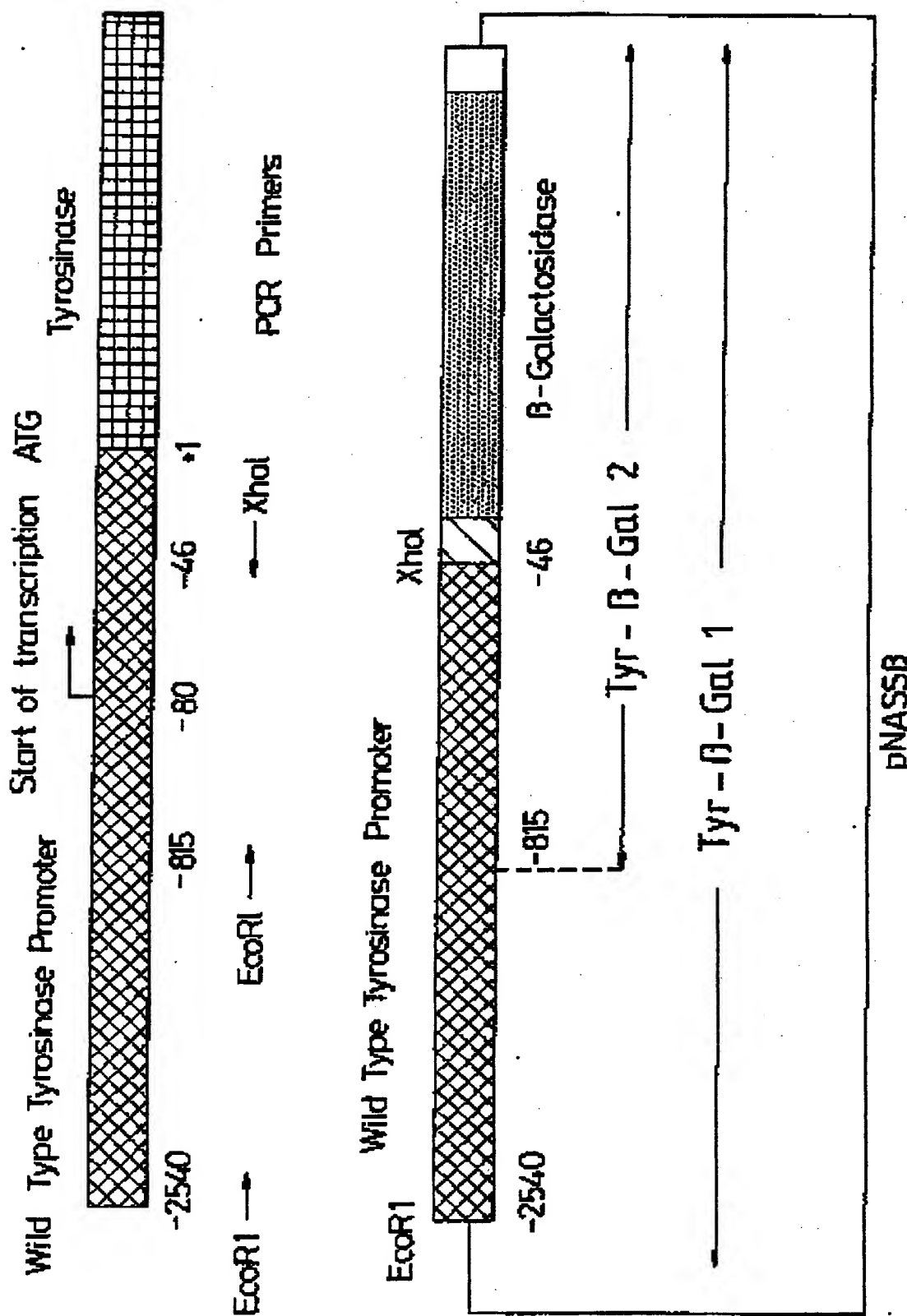


Fig. 1 (PAGE 2 of 3)

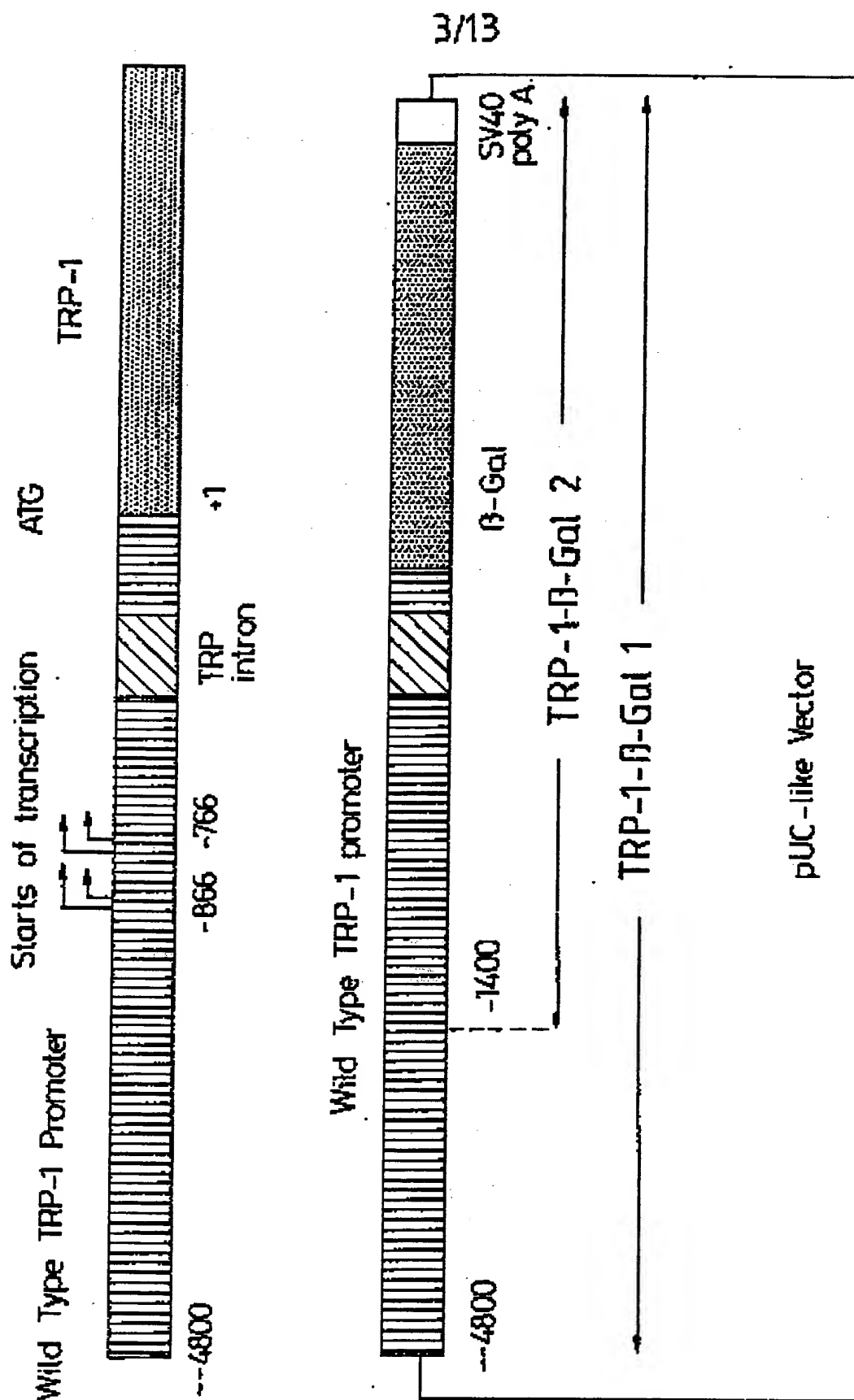
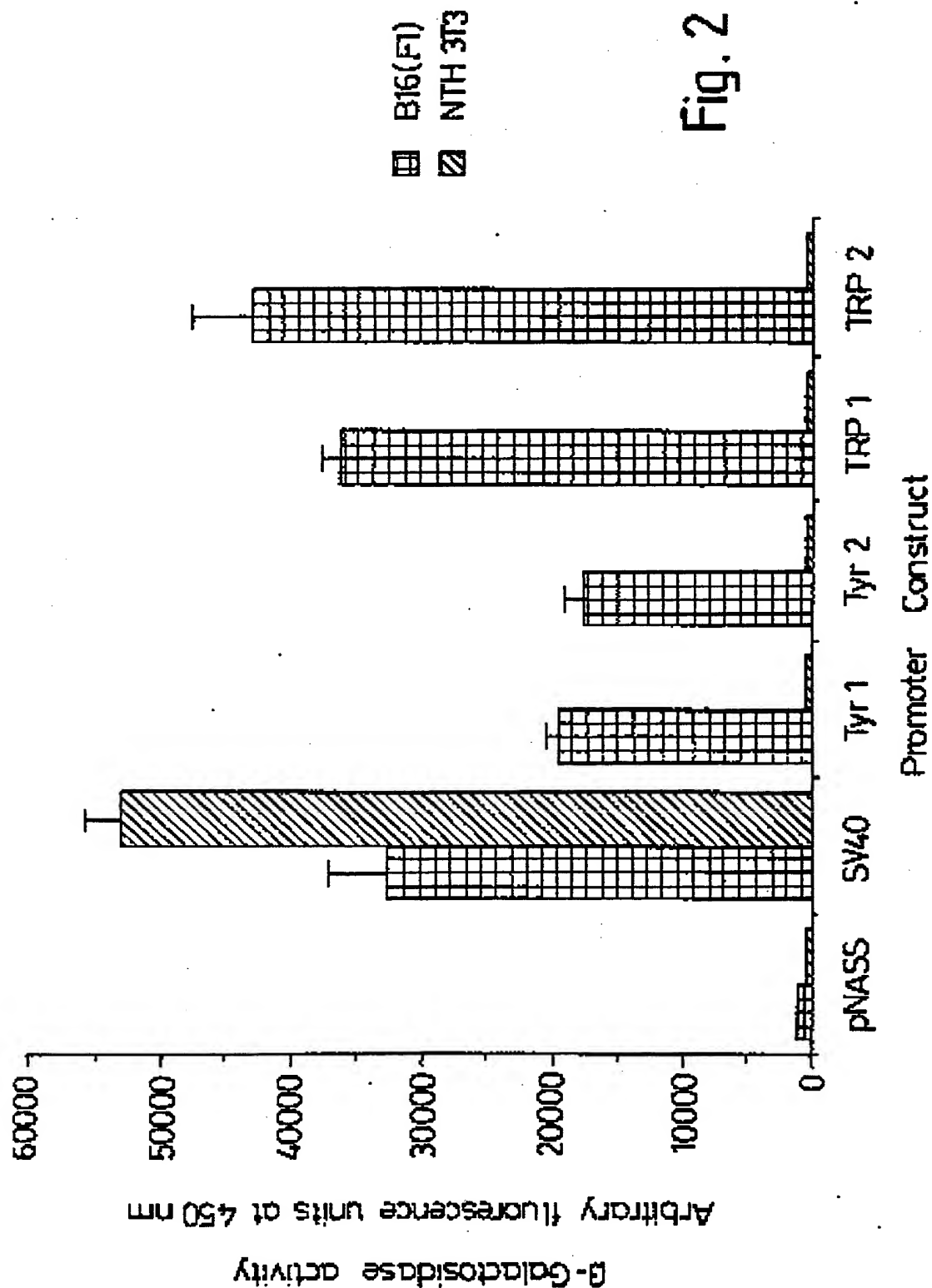
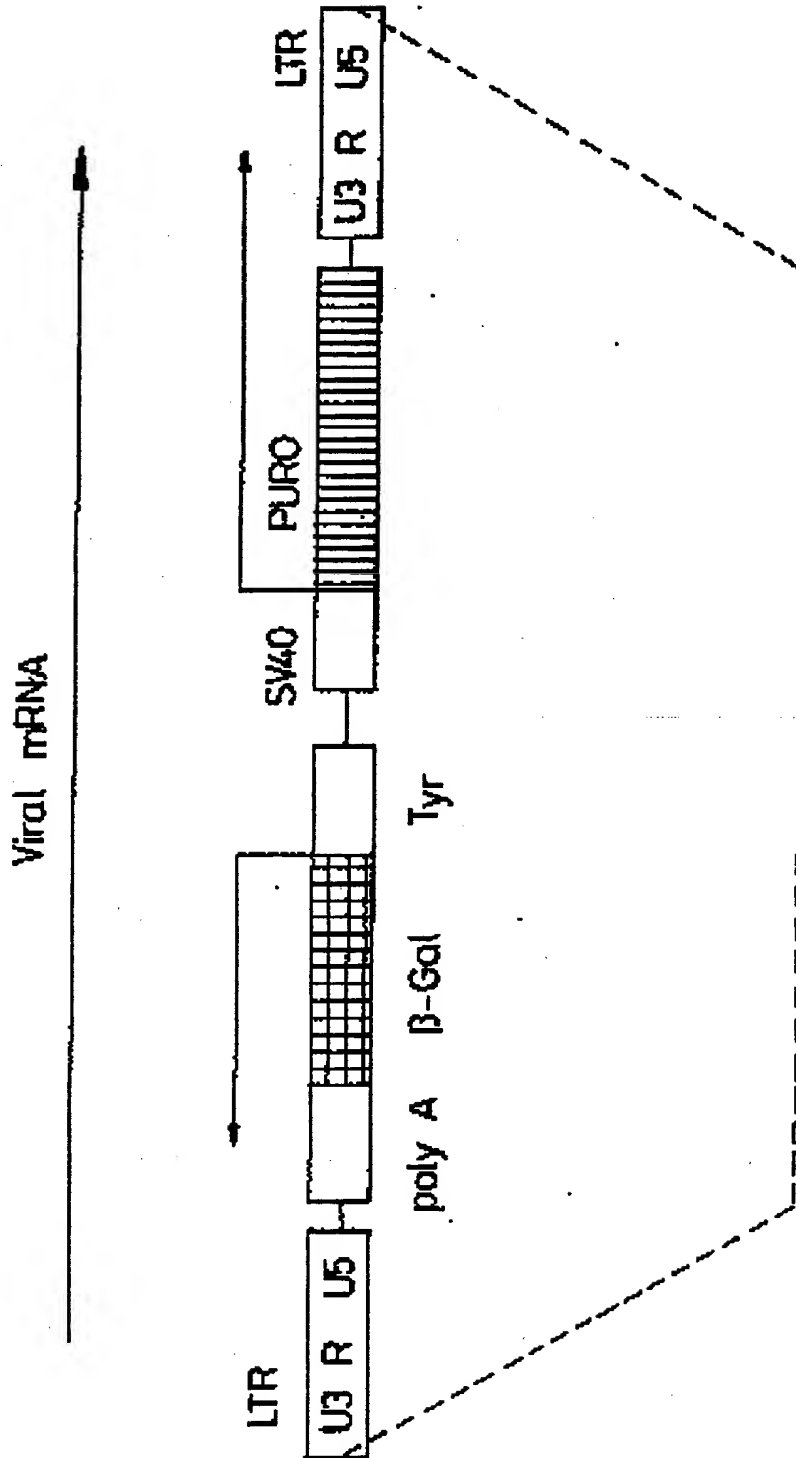


Fig. 1 (PAGE 3 of 3)

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INDUSTRIAL SHEET

Fig. 3

pBabe Puro (Tyr-β-Gal)

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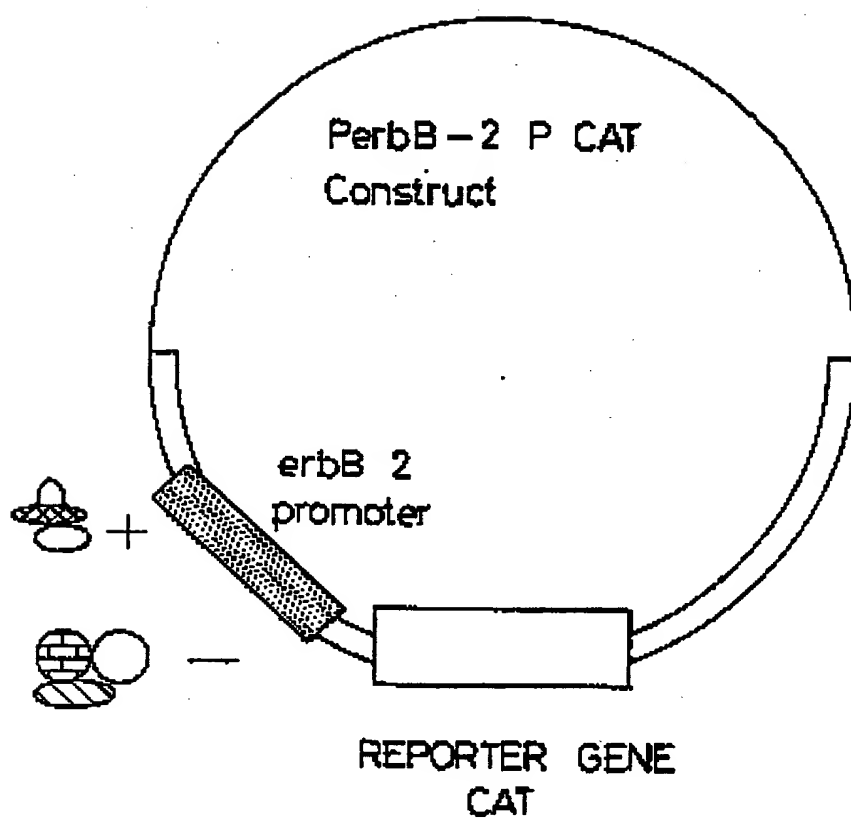


Fig. 4

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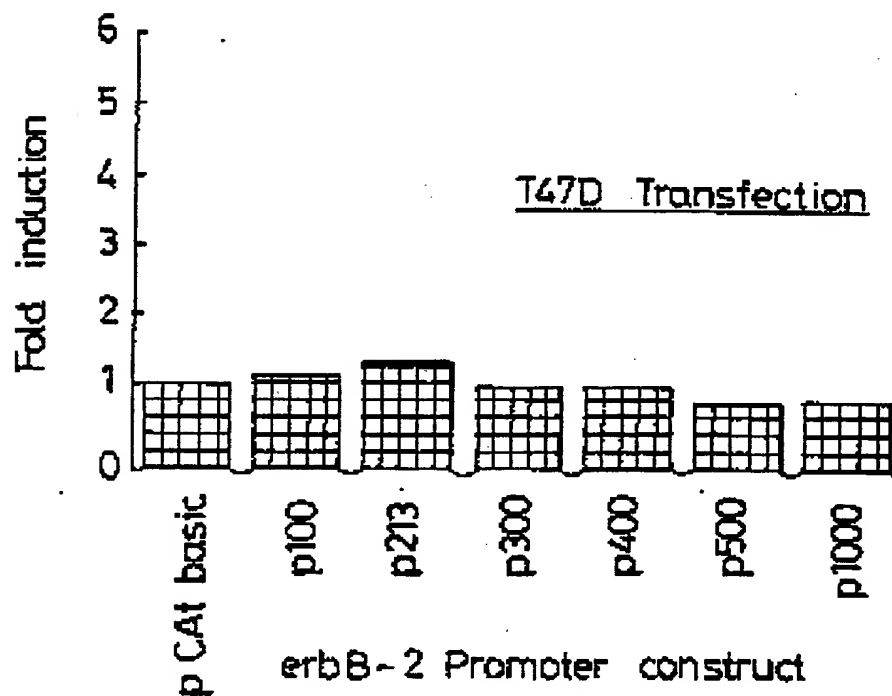
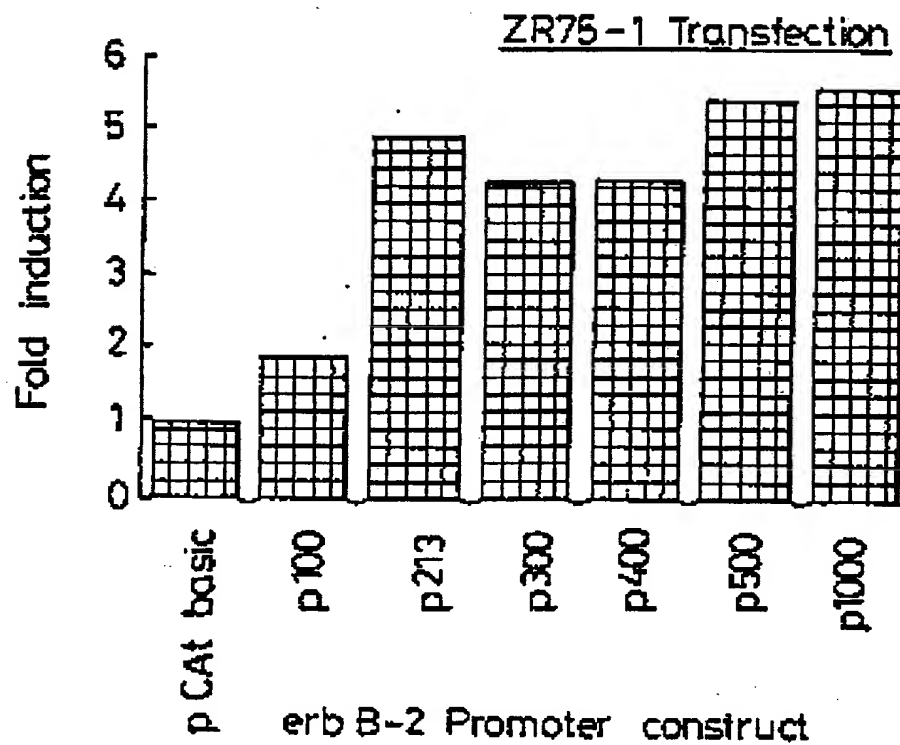


Fig. 5

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-787 CGAGCGCCCTCAGCTTCGCGCCAGGCCGCAAGGCTCCCGGTGACCACTAGAGGGCGGAGGAGCTCTCTGGCCAGT GC Box
 -707 GGTGGAGAGTGGCAAGGAGGACCCCTAGGTTTCATCGGAGCCGAGGTTTACTCCCTTAAGTGGAAATTTCTTCCCCCCT
 -627 CCTCCTTGGCTTTCPCCAAGGAGGAACCCAGGCTGCTGGAAAGTCCGCTGGGGCGGGGACTGTGGTTTCAGGGGAGAA Sp1
 -547 CGGGGTGTGGAACGGGACAGGAGCGGTTAGAAAGGTGGGCTATTCCGGGAAGTGGTGGGGGAGGGAGCCCAAACTA MPBF
 -467 GCACCTAGTCCACTCATTTATCCAGCCCTCTTATTCTCGGCCGCTCTGCTTCAGTGGACCCGGGAGGGCGGGAGGTGG GC Box
 -387 AGTGGAGACCTAGGGGTGGCTTCCGACCTTGCTGTACAGGACCTCGACCTAGCTGBCCTTGTTCCCAATCCCCACGT
 -307 TAGTTGTGCCCTGAGGCTAAACTAGAGCCCAAGGGCCCCCAAGTTCAGACTGCCCTCCCCCTCCCCCGGAGCCAGG
 -227 GAGTGGTTGGTGAAGGGGAGGCCAGCTGGAGAACAGGGGTAGTCAGGGGGTTGAGCGATTAGAGCCCTTGTAACCT
 -147 ACCCAGGATGGTTGGGAGGAGGAGGAGAGGTAGGAGGTAGGGGAGGGGGGGGTTTGTTCACCTGTACCTGCTCG Sp1 E-MUC1
 -67 CTGTGCTTAGGGCGGGCGGGGAGTGGGGGACCGTATATAAGCGGTAGGCGCTGTGCCCGCTCCACCTCTCAAGC GC Box
 +14 AGCCAGGCCCTGCTGATCTGTCTGTGCCCCCTCCCCACCCATTTCACCACCCACCATG
 ↑
 -33

Fig. 6

-1571	NcoI ↓ CATGGTGTCCGACTTATGCCCAGAGAGATGTTGAGCAAACTTATCGCTTATCTGCTTCTCATAGAGTCTT
-1501	CCAGACAAACTCGCGAACTCGTGCAAGGTAGGCGGATCTGGGTGCGACCTGCAGGTCAACGGATCCCTTCT
-1431	TGACCAAGTATAGCTGCATTCTTGGCTGGGGCATTCCAACTAGAACTGCCAAATTTAGCACATATAAATAA
-1361	GGAGGCCCCAGTTAAATTTGCAATTCAGATAAACCAATGAATAATTTGTTAGTATATAATGTCTCCATGCA
-1291	TATCTTGTGAAANTTAAAAAATAAAGTCTTCTTCCTTCATGCCCCACCCCTACCACCTAGGCCCTAAGGANTAG
-1221	GGTCAGGGGCTCCBAATAGCAATGTGGTTCAGAACTGGAATTAAGCAGGCTAATAGAAAGCAAGGGGCAAA
-1151	GAGGAAACCTTGARTTCATTGGGTGCTGGGTGCTCTCTTAAATTAAGCAAGAGGGTGCATTTTGAAGAAT
-1081	TGAGATAGAAAGTCTTTTGGCGCTGGGTGCAGTTGCTGGTGGTGTGTAATTCAGACACTTTTGGGAGCCCTAG
-1011	GCGGGAGGATCACTCTGAGGTTCAGAGGTTCAAGACAGGCTCACCAACCTGGAGAAACCTGTCTTTACTAA
-941	AAATACAAAAAATTTCAGCTGCTCATGGTGGCACATGCCCTGTAAATCCAGCTGCTCGGAGGCTGAGGCCAG
-871	GAGAAATCACTTGAACCCAGGAGGCAGAGGTTGTGGTGAGCAGAGATCGGGCCATTCCTCTCCAGCCTGGG
-801	CAACAAGAGCAAAAGTTGCTTTAAAAAATAAAGTCTTTGSAATGTGACTGTCTCTCTCCCAATTTC
-731	TAGACCTCTTTAAGATCATGCTTTTCAGATXACTTCAAAAGATTCCAGAAAGATATGCCCGGGGCTCTGGA
-661	AGCCACAAGGTAACAACAACAATCCCCCTCTTGACTATCAATTTTACTAGAGGATGNGTGGGAAAC
-591	CATTATTGATAATTAACAATAAGCTTTGGCATGGAGTAGGATGCAGGCTCCCAAGGATTTAAGAT
-521	AAAACTGAGGACTTAAAGGGTGTAAAGAGTGGCAGCCTTAGGGAATTTATCCGGGACTCCGGGGGAGGGG
-451	GCAGAGTCAACAGCCTTGATTAAGGATTCCTCGAGGAAAGTGTGACAAACGCTCCAGGGCAACCCAG

FIG. 7 (PAGE 1 OF 2)

2. IDENTIFY THE SUBJECT

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```

-381  GCGTCCGGGGCTAGGAGGGA CGACCCAGGCCTGCGCGAAGACAGAGGGAGAAAGTCAAGCTGGGAGTTGCC
-311  GACTCCCAAGACTTCGTTGGAAATGCAATTGAGAGGGGCGAGCTGCGAGCGCGCTTGCTCCGAAATCACAGGA
-241  GAAGGAGGAGGTGGAGCAGGAGGGCTGCTTGAGGAGAGTATTAAGAAATGAAGTTCTGCAAGCTGAGATTCCCC
-171  TCCATTGGGACCGGAGAAACCAAGGGGAGCCCCCGGCGAGCCGCGCCCCCTTCCCAAGGGGCCCTTTAC
-101  TCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
-31  GAGCCATGGGGCGGAGCGCGAGTGAAGCAACCAATGGAGCTGG
      |
      NCOI
      -1

```

FIG. 7 (PAGE 2 OF 2)

1. A method of determining the sequence of a nucleic acid molecule, comprising:

Cerb B3 promoter

BamHI	SmaI	FP/A	FP/B	
GGATCCGTC	CCGGGACTAGC	AGGGCTNTGGG	CAGCAACCCG	CAGGAGCCGCTCTGGCCAGGTCC
1				70
			OB2-1	
				71
				140
				210
				280
				350

Fig. 8 (PAGE 1 of 3)

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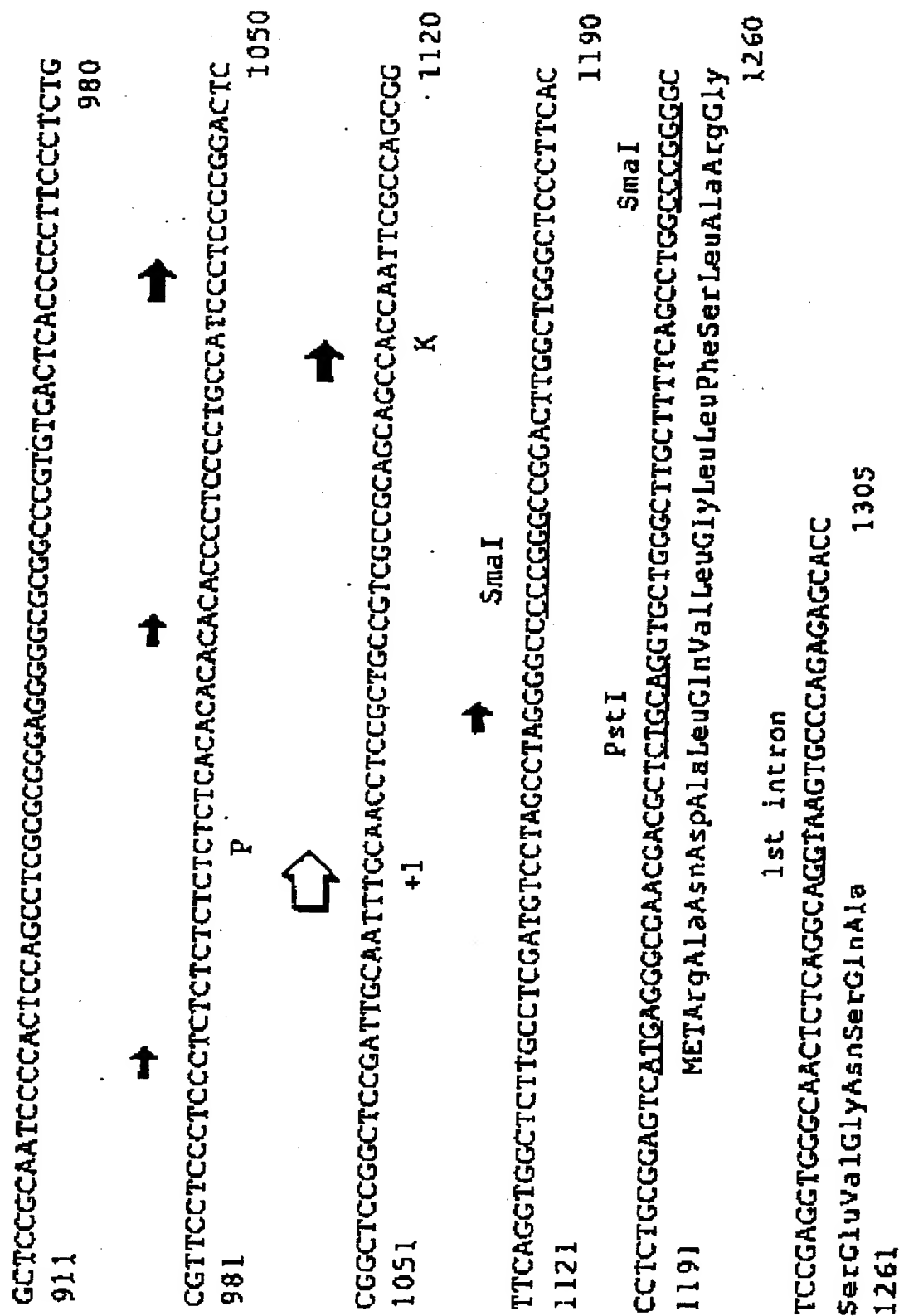


Fig. 8 (PAGE 3 of 3)